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The interaction of phagocytic cells with immune complexes in rheumatoid arthritis.

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THE INTERACTION OF PHAGOCYTIC CELLS WITH IMMUNE COMPLEXES IN RHEUMATOID ARTHRITIS

Submitted by

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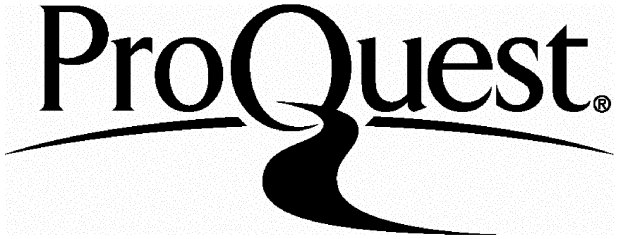

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This thesis is dedicated to my parents,

Leslie and Phyllis Minty.

I should like to acknowledge the helpfulness and extreme patience of those with whom I worked, and wish to thank all the staff and patients of the Royal National Hospital for Rheumatic Diseases who so freely donated their blood and without whom this work could not have been undertaken.

Abbreviations Used in the Text and Figures

ACA	anti-complementary activity
BSA	Bovine serum albumin
Clq	the 'q' subcomponent of the first component of complement
C3	the third component of complement, activated after C_{142} in the classical pathway
DNA	deoxyribonucleic acid
EA	Egg albumin
EBSS	Earles Balanced salt solution
FCS	foetal calf serum
HAGG	heat-aggregated immunoglobulin G.
HI	heat-inactivated (serum)
IC	immune complexes
IgG	immunoglobulin G
IgM	immunoglobulin M
LDH	lactic dehydrogenase
M199	tissue culture medium 199
MEP	monocyte-enriched population
MNC	mononuclear cells
MNP	mononuclear phagocytes
NSAID	non-steroidal anti-inflammatory drugs
PB	peripheral blood
PBS(A)	phosphate buffered saline (A, with egg albumin 2%)
PMN	polymorphonuclear leucocytes
PGU	purpurogallin units
RA	rheumatoid arthritis
RF	rheumatoid factor (MxRF:IgG and IgM RF preparation)
SD	standard deviation

SF	synovial fluid
SH	thiol group
SLE	systemic lupus erythematosus

SUMMARY

Polymorphonuclear leucocytes and monocytes play an important role in the inflammatory response. The ability of these cell types to degranulate in response to particulate aggregated IgG1 was studied after their isolation from the peripheral blood of two groups of people. These consisted of patients suffering from the chronic inflammatory disease Rheumatoid Arthritis, and, for comparison, a number of apparently healthy, normal controls. The patients' neutrophils manifest a lower responsiveness than normals in this system, while the monocytes behaved similarly in both groups. The membrane-mediated "metabolic burst" was measured directly by superoxide release and serum thiol oxidation; in neither of these was a significant difference found between normals and the patients, although patients' cells showed a trend towards a higher thiol-oxidising ability. Thus a degranulation or lysosomal defect is implicated.

The effect of the addition of rheumatoid factor to the incubation medium was studied. A preparation which consisted of both IgM and IgG anti-IgG (MxRF) was found to inhibit degranulation by monocytes, while pure IgM RF had no such effect. A similar, though less marked, inhibition could alternatively be induced by adding soluble aggregates of IgG1 to the degranulation-inducing system. The implications of this are discussed. The soluble aggregates and the MxRF, whilst not causing degranulation, were able to stimulate neutrophils and monocytes to release oxygen radicals. This confirms previous observations of the independence of the two reactions.

CHAPTER 1. INTRODUCTION

In a progression of experiments from 1882 to the opening years of the twentieth century, the now-famous scientist Elie Metchnikoff showed an awareness of the importance of phagocytes in inflammatory responses (1883, 1938). He was able to distinguish between microphages and macrophages and noted that the former preceded the latter in moving into a site of inflammation. The aim of this introduction is to put forward some modern knowledge about these cells and relate it to their role in acute and chronic inflammation.

Mononuclear phagocytes

It is now realised that the macrophages that accumulate in inflammatory areas are of monocyte origin, and these two cell types form the major part of what is now termed the mononuclear phagocyte system (van Furth, Raeburn and van Zwet, 1979). The bone marrow monoblasts and promonocytes, which can divide, are also included. The peripheral blood monocyte is derived from these precursors and has an apparent half-life of seventy-one hours in the circulation. It has a diameter of about 14 μm and staining with Romanovsky dyes reveals a large blue-grey, horseshoe shaped, nucleus against a pale grey cytoplasm in which no granules can be distinguished. Vacuoles can be present. Figure 1a illustrates the typical appearance seen in a smear of peripheral blood. The cell is peroxidase and non-specific esterase positive (Yam, 1971), and both properties are useful in its resolution from the smaller, non-phagocytic, lymphocyte. Following its circulation, the monocyte migrates to a tissue site to develop into a macrophage, which has a smaller, rounded and

purple-staining nucleus and a large amount of blue cytoplasm in which vacuoles are present. (see Figure 1b, which shows a number of alveolar macrophages harvested by broncholavage). The cell diameter is about 20 μm.

The mononuclear phagocyte possesses an extensive lysosomal system and secretory capacity (Nathan,Murray and Cohn, 1980). Since the circulating monocyte is relatively non-activated, it contains fewer enzymes than its tissue counterpart (Bennet and Cohn, 1974), and has a higher nuclear:cytoplasmic ratio. The level of lysosomal enzymes differs amongst macrophages, and tends to show a positive correlation with their activated state (Cohn, 1978), although some cultured macrophages appear to lose their peroxidatic activity (van der Meer *et al.*, 1979). One function of this armamentarium is to inactivate or kill ingested particles within the protective membrane of a phagosome. The products that macrophages can be induced to secrete into the environment include neutral proteases such as plasminogen activator (PA), elastase and collagenase; complement components; enzyme inhibitors and chemotactic factors (Davies *et al.*, 1977; Steinman and Cohn, 1974). PA promotes activation of the fibrinolytic system, while collagenase influencescollagen deposition in the extracellular space, and elastase can create chemotactic fragments from the complement component C5 (Ward, 1979).

In addition to their role as secretory cells, macrophages are important in immune responses; effective antigen presentation at the macrophage surface is a requirement for most lymphocyte responses to antigen (McConnell, 1976b), whether for the production

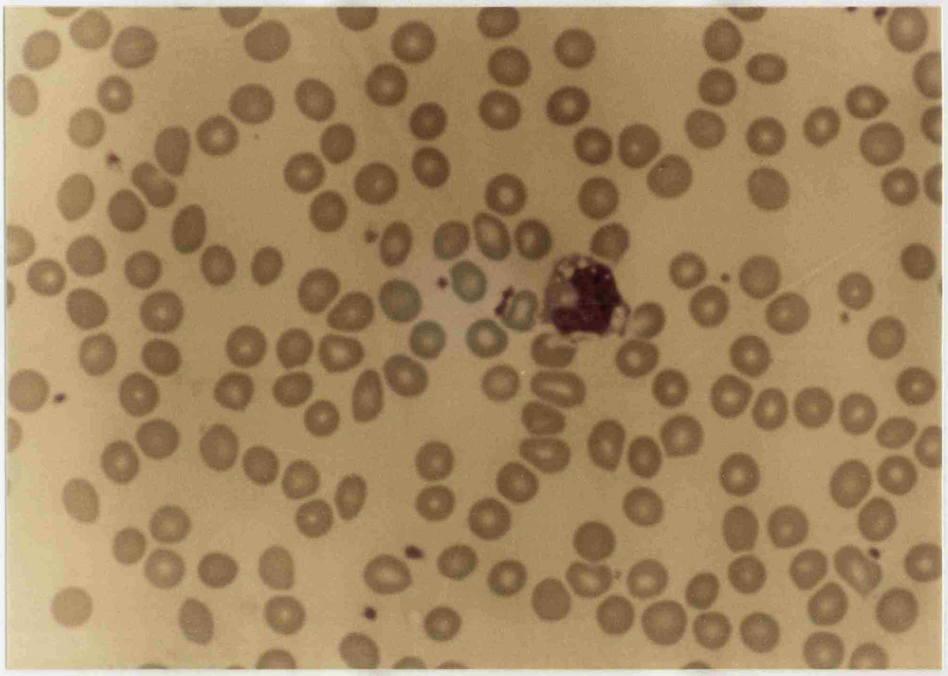


Figure 1a.
Peripheral blood monocyte from normal control. x630

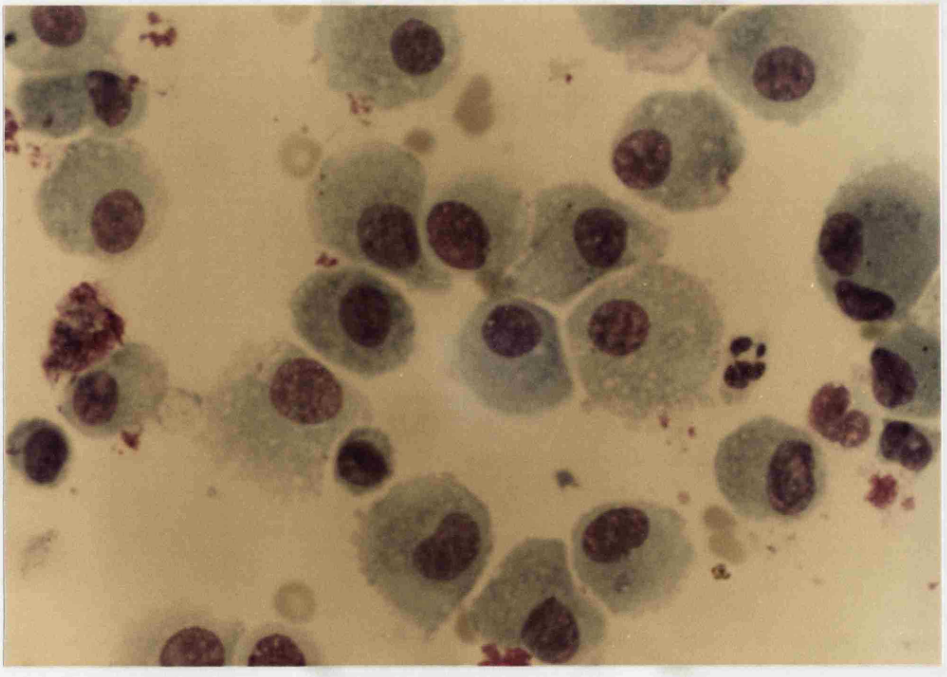


Figure 1b.
Alveolar macrophages, obtained at bronchoscopy with lavage. To be noted are the binucleate cells and the relative size of macrophage and monocyte (see Figure 1a). From a patient with scleroderma. x 630

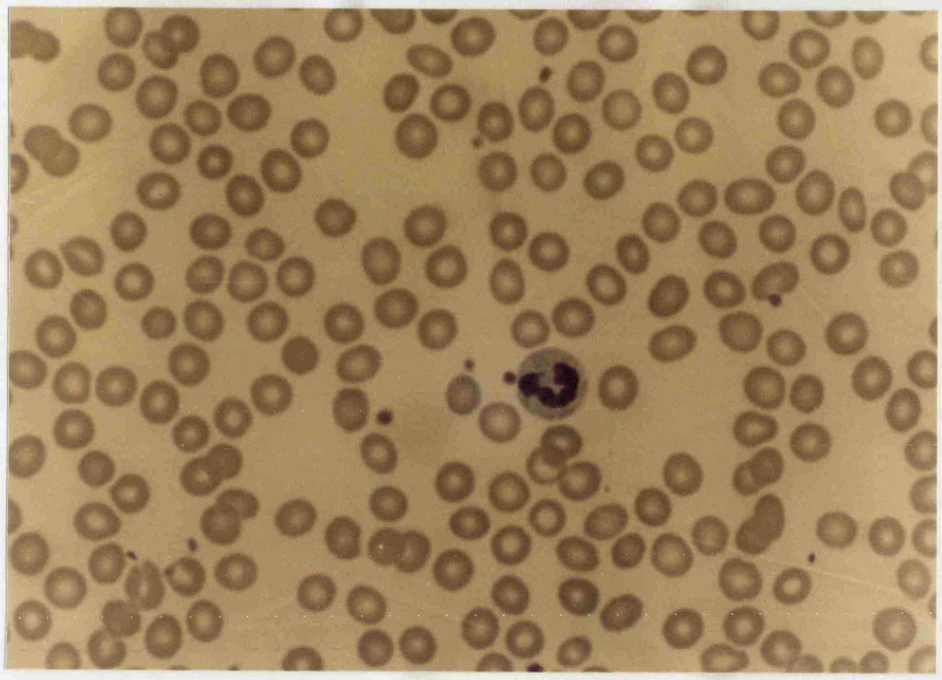


Figure 1c.

Peripheral blood neutrophil. ⌊——⌋ represents size of a monocyte for comparison (Figure 1a). Each erythrocyte is approximately 7 µm. From a normal control. x 630

of antibodies or for cell-mediated immunity (Nelson, 1981). The intercalation of these diverse properties has been stressed in, for instance, the host response to herpes simplex virus, as has been reviewed by Morahan and Morse (1979).

Surface receptors on mononuclear phagocytes.

Human monocytes express receptors for the Fc portion of IgG1 and IgG3 (Huber and Fudenberg, 1968), but only weakly for IgG2. They also have receptors for IgM and for the complement component fraction C3b(Ehlenberger and Nussenweig, 1977). Although they were thought to possess C3d receptors (Ehlenberger and Nussenweig, 1977), it now seems likely that this was confused with the C3b' receptor, as discussed by Scott (1981). These recognition sites enable the cells to bind and phagocytose opsonized particles. The immunoglobulin receptors may also be important in the role of monocytes as accessory cells in T-B lymphocyte cooperation (Roitt, 1977).

Polymorphonuclear leucocytes.

In contrast to the bimodal behaviour of the macrophages, the cells that Metchnikoff called microphages are solely phagocytic. These are now termed granulocytes or polymorphonuclear leucocytes (PMN) because of the granules and multi-lobed nucleus discernable after fixation and staining with Romanovsky dyes (see Figure 1c). There are three populations of these bone marrow derived cells which, in man, are neutrophilic (about 95%), eosinophilic (4%) and basophilic (1%). It is the neutrophils which are employed in host defence against bacterial invasion, and play a large part

in inflammatory responses, be they mediated by infection or by tissue damage. Eosinophils are prominent in counteracting parasitic infection (Glauert *et al.*, 1978), and basophils are probably precursors of mast cells.

The neutrophils are smaller than monocytes (approximately 10 µm in diameter), and this can be seen if comparison is made between Figures 1a and 1c; an erythrocyte is about 7 µm across. The cytoplasm stains faintly pink owing to the presence of a large number of granules. The migrating cell is not round; when allowed to move over a glass slide its shape changes constantly, with a broad pseudopod preceeding a region containing the nucleus and granules, and a smaller, trailing portion. Its adhesive properties and ease of motility (Zigmond, 1978) are indicative of the function of the cell; that is, to stick to and proceed through the capillary endothelium to the tissues in response to chemotactic factors. In contrast to the monocyte, the neutrophil does not apparently marginate in the absence of a stimulus, nor does it mature once in the tissues. Its lifetime is about ten hours in the blood and up to two days in the extra-vascular regions, though 3 - 4 hours seems the modal lifespan (Bainton, Ullyot and Farquar, 1971; Hirsch, 1974). The fate of the effete cells in the blood is unknown.

The granules from various species have been characterized as membrane-bound vesicles which develop during the maturation of the granulocyte in the bone marrow. In humans they have been resolved into azurophil and specific granules; the former contain

all the cellular myeloperoxidase, the acid hydrolases and part of the lysozyme, and the latter contain the remainder of the lysozyme and lactoferrin. Alkaline phosphatase and an acid phosphatase were found to be associated with a membranous rather than a granular fraction (Bretz and Baggiolini, 1974). More recent work by Segal and his colleagues has suggested that the peroxidase and acid hydrolases are located within different lysosomes (Segal, Dorling and Coade, 1980). The granules as a whole contain proteases, carbohydrases, lipases, oxidases and enzymes able to cleave phosphates, sulphates and nucleic acids. Other constituents include lactoferrin and cationic proteins. (Hirsch, 1974; Oseas *et al.*, 1981). Cytochemically, neutrophils stain very strongly for peroxidase and for an esterase that is distinct from the monocytic enzyme (Yam, 1971; van Furth, Raeburn and van Zwet, 1979).

Both polymorphonuclear and mononuclear phagocytes have a bactericidal capacity which is dependent on the generation of highly reactive oxygen metabolites in response to the stimulus. The superoxide ($O_2^{-\cdot}$) forming enzyme which may initiate the reaction (NAD(P)H-dependent $O_2^{-\cdot}$ generating enzyme) is membrane-bound and appears to be associated with the plasma membrane rather than with specific or azurophil granules (Dewald *et al.*, 1979), as are the active serine proteases that appear to be necessary for initiation and maintenance of the response (Kitagawa, Takaku and Sakamoto, 1980a). Experiments conducted by McPhail (McPhail, Henson and Johnston, 1981) suggest that the cell is switched on by multiple mechanisms. Evidence is in favour of the oxidase

being a transmembrane protein with the reducing moiety on the cytoplasmic side (Babior *et al.*, 1981); thus when the phagocytic vacuole is formed the interior contains the superoxide (and hydrogen peroxide) molecules while the cytoplasmic face supplies the hydrogen ions from an HMPS (hexose monophosphate shunt) that has also been activated as part of this coordinated set of events. Another enzyme, catalase,can mediate the breakdown of hydrogen peroxide to water and oxygen, with two more active molecular species, singlet oxygen and hydroxyl radical, as intermediates (Weiss, King and LoBuglio, 1977; Rosen and Klebanoff, 1979). Myeloperoxidase (MPO) from lysosomes uses hydrogen peroxide and a halide in order to halogenate, and kill, bacteria (Klebanoff, 1975; Rosen and Klebanoff, 1979).

The characteristics of this burst of activity have been detailed by Klebanoff and co-workers (Klebanoff, 1975; Rosen and Klebanoff, 1976 and 1979), and are illustrated in Figure 1.2, which is based on a scheme proposed by Roos, Weening and Loos (1979). These authors have stressed the importance of another peroxidase system (GSH-peroxidase/GSSG-reductase) which stimulates the HMPS when hydrogen peroxide is present, while detoxifying the latter by allowing it to oxidise the reduced glutathione (Roos, Weening and Loos, 1979). GSH and GSSG are the reduced and oxidised forms, respectively, of the peptide glutathione.

Inherited enzyme deficiencies have helped to determine the relative importance of the reactions for bacterial killing, for instance in individuals suffering from catalase deficiency and

Figure 1.2. Reactions involved in the generation of highly reactive oxygen metabolites

Abbreviations employed:

Cyt C Fe^{3+}	ferricytochrome C
GSSG and GSH	oxidised and reduced form of glutathione
Glu-6-P	glucose-6-phosphate
G-6-P deHse	glucose-6-phosphate dehydrogenase
6-P-Gluc	6-phospho-gluconate
6-P-Gluc deHse	6-phospho-gluconate dehydrogenase
Rib-5-P	ribulose-5-phosphate
NAD(P),	nicotinamide adenosine dinucleotide (phosphate): with H, reduced form
H_2O_2	hydrogen peroxide
$OH^{\cdot}$	short-lived hydroxyl radical
$O_2^{-\cdot}$	superoxide
$^1O_2$	singlet oxygen

Reactions:

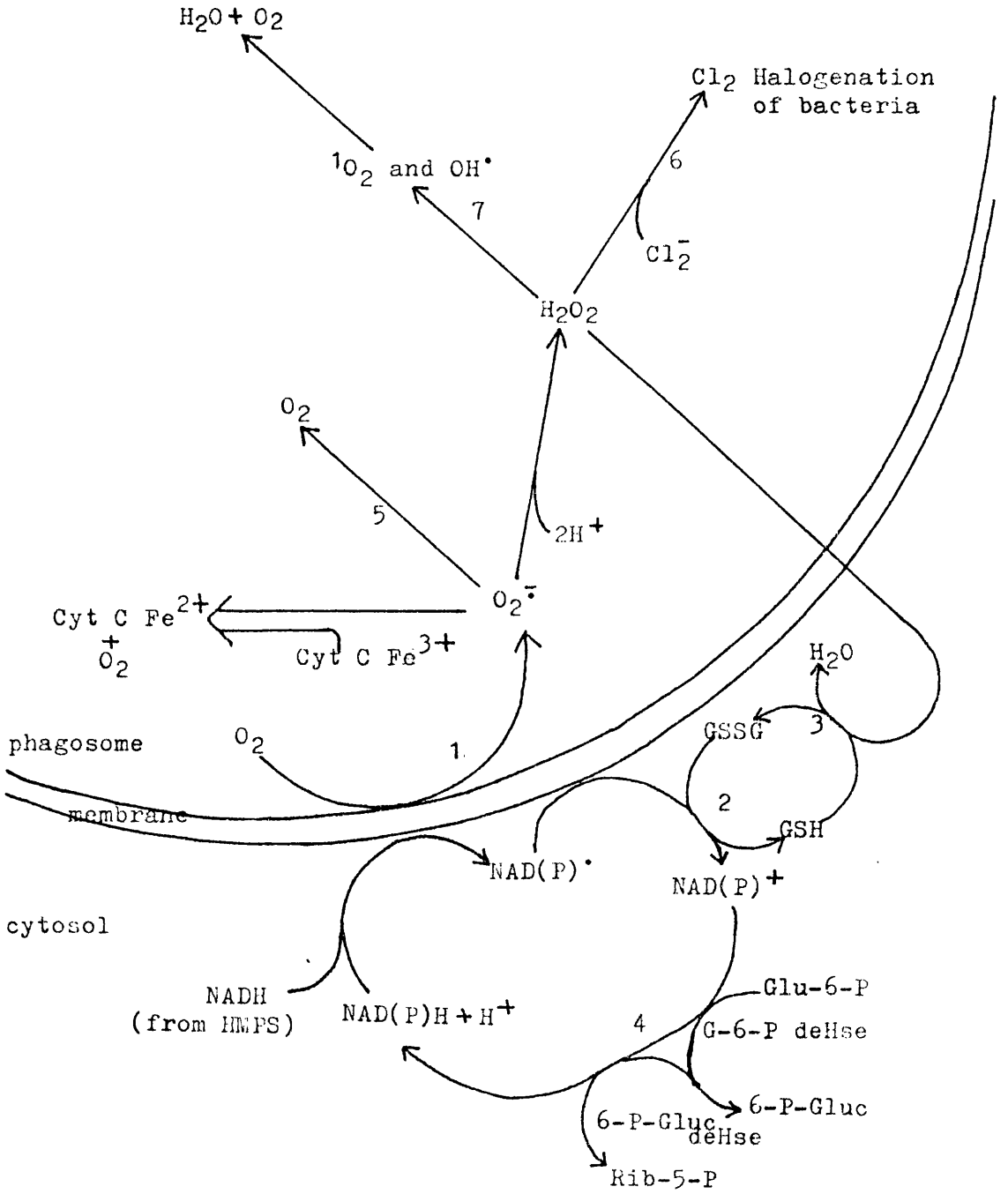
1. NADPH-dependent O_2-generating enzyme (NADPH oxidase)
2. Glutathione reductase
3. Glutathione peroxidase
4. Reactions of the hexose monophosphate shunt (HMPS)
5. Superoxide dismutase. The cytochrome C reaction predominates if present (experimental only)
6. Myeloperoxidase
7. Catalase

facing 13

Figure 1.2

Reactions involved in the generation of highly reactive oxygen metabolites.

See legend opposite

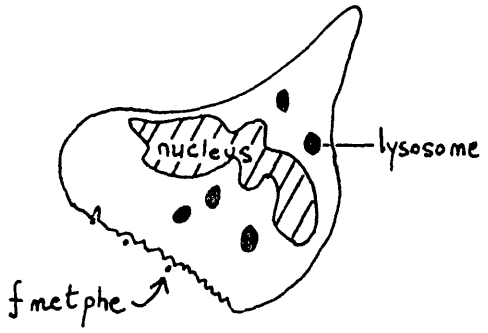


from chronic granulomatous disease (Klebanoff, 1975; Rosen and Klebanoff, 1976; Roos *et al.*, 1980). Acatalasemic individuals do not suffer from an increased indidence of infection, in contrast with which is the paucity of bacterial killing by CGD neutrophils through lack of activation of the membrane oxidase. Non-peroxide producing bacteria are the major source of infection in this disease (Roos *et al.*, 1980; Baehner and Boxer, 1979).

The release of reactive oxygen species makes possible the reduction of nitroblue-tetrazolium (Wenger and Bole, 1973) and ferri-cytochrome C (Kitagawa, Takaku and Sakamoto, 1980a; Salin and McCord, 1974) and the excitation of luminol and lucigenin (Easmon *et al.*, 1980; Williams and Cole, 1981b) and these assays have been used to good effect to assess this release. The stimulation occurs similarly in both human granulocytes and monocytes (Sagone, King and Metz, 1976) and within one minute of exposure to the stimulus, if measured by chemiluminescence (Williams and Cole, 1981b), although the lag phase is more prolonged if cytochrome C is used as a detector (Rosen and Klebanoff, 1976). Malawista and Bodel's work in 1967 (Malawista and Bodel, 1967) showing that increased oxygen consumption could be dissociated from phagocytosis appear to have been confirmed by McPhail (McPhail, Henson and Johnston, 1981) and Williams and Cole (1981b) who have demonstrated by cytochrome C reduction and chemiluminescence, respectively, a response to the chemotactic peptide fMLP (f-Met-Leu-Phe) and other 'soluble' compounds which do not promote a phagocytic event which leads to degranulation. These small molecules may induce pinocytosis and activation of the membrane signals.

The peptide chemotactic factors do certainly activate a putative phagocytic mechanism because treatment of neutrophils with cytochalasin B reveals the secretogogue activity of the factors. Cytochalasin B is thought to act by disrupting microfilaments within the cell and allowing greater movement of granules. The larger protein chemotaxins do not need this fungal metabolite in order to stimulate release of enzymes from the cell into the environment (Wilkinson and Bradley, 1981), probably because they bind well to the substratum. Henson and his associates (Henson, Johnson and Spiegelberg, 1972) demonstrated that soluble immunoglobulin aggregates (both thermally and chemically induced) did not cause enzyme release from neutrophils in suspension, but did do so when bound to filters to form a non-phagocytosable surface; phagocytosis is a surface phenomenon since neutrophils crawl rather than swim (Henson, 1977). Exocytosis occurred in the fluid phase when similar, but insoluble, aggregates were incubated with neutrophils (see also Treadway *et al.*, 1979). Both soluble and insoluble aggregates promoted HMPS stimulation. The possible events in this differential lysosomal discharge are shown in Figure 1.3.

When human granulocytes are allowed to migrate, in a Boyden chamber, towards casein, they release a chymotrypsin-like cationic protein from their granules which has multiple effects on cell behaviour, especially on migration, aggregation and phagocytosis (Venge, 1979; Bockenstedt and Goetzl, 1980). The same group of proteins was assigned a different role by Klebanoff (Klebanoff, 1975) who noted their antimicrobial activity, especially in the heterophiles from species (other than human) which do not contain

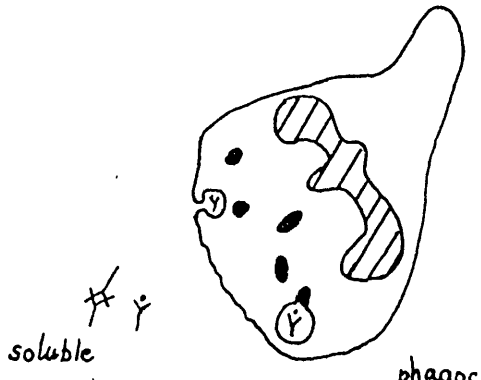


pinocytosis

Binding

↓

metabolic excitation



soluble aggregate or complex

phagocytosis

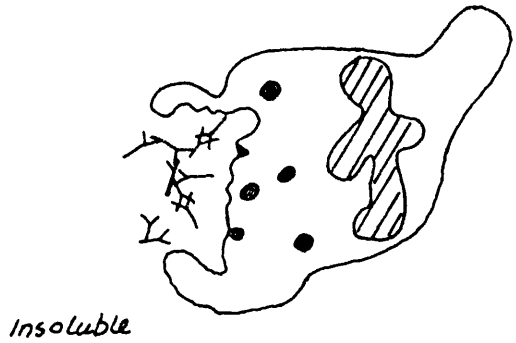
microtubule reorganization

↓

Phagosome formation and movement of lysosomes

↓

Fusion



insoluble aggregate or surface-bound complex

Frustrated phagocytosis

Phagosome not closed off

↓

Exocytosis

Figure 1.3 The response of phagocytes to different stimuli.

myeloperoxidase; and yet another function in coagulation, by Goldstein (1974). These distinct properties need not be mutually exclusive since the compounds are themselves heterogenous (Olsson and Venge, 1974). Congenital defects in cationic proteins might clarify the situation but they are heterogeneous, and one has not yet been described (Klebanoff, 1975). Known deficiencies in lysosomal enzymes are not confined to the bactericidal failures mentioned above: the absence of any one of the acid hydrolases will result in the accumulation, within the phagocyte, of the substrate e.g. a mucopolysaccharide, to the detriment of and early death of the sufferer (Zurier,1979) . A primary defect may occur elsewhere in the cell, for instance in the Chediak-Higashi syndrome in which the microtubule assembly is aberrant (Zurier, 1979): there is subnormal degranulation and bactericidal activity since the fusion of the phagosome and lysosomes is necessary for myeloperoxidase and/or cationic proteins to effect killing of an ingested organism, and for hydrolytic enzymes to subsequently degrade the foreign body.

Surface receptors on neutrophils.

Human neutrophils have receptors for all the IgG and IgA subclasses but these immunoglobulins bound to antigen may be relatively inefficient at promoting phagocytosis. They do not have receptors for IgM (Ehlenberger and Nussenweig, 1977). Binding of the complement component C3 to the antigen probably promotes contact between phagocyte and particle, but ingestion by neutrophils does not occur unless IgG, or IgA, is also present (Ehlenberger and Nussenweig, 1977; Henson, 1977). Both monocytes and neutrophils

have receptors for C3b, but apparently not for C3d which is a breakdown product of the latter. Another biproduct, C3b', appears to be bound by neutrophils (Scott, C.S., 1981), and also by monocytes (see a previous section). When another complement component is cleaved, the product C5a is a potent anaphylatoxin and highly chemotactic for neutrophils. Its physiological end-product, $C5a_{\text{des Arg}}$ loses the former property while retaining the latter (Henson, 1977; Chenoweth and Hugli, 1980) although to a lesser extent under some *in vitro* conditions (Perez *et al.*, 1980). Removal of more C-terminal amino acids abrogates this activity although it can still bind to the cell, which reinforces the principle that occupation of a receptor does not necessarily activate the phagocyte. Neutrophils also possess receptors for synthetic N-formylmethionyl peptides and for more natural compounds (Williams *et al.*, 1977; Spilberg and Mehta, 1979; Wilkinson and Bradley, 1981).

Phagocytic cells; their role in inflammation

Insight into the cellular and chemical changes induced by acute inflammation has been gained using experimental systems such as the Reverse Passive Arthus reaction which is immune complex mediated and the reaction to carrageenan. These changes show marked similarities although the exact kinetics may vary. The stimulus *in vivo* may be tissue injury, which would promote platelet clumping and release of serotonin (5-hydroxy-tryptamine), as well as activation of the coagulation cascade. It may be the presence of bacteria and the endotoxins that they can secrete (Gram positives only) (Ebert and Grant, 1974) which can damage leucocytes and also activate C3

or cell fragments left after the replication and successful maturation of a lytic virus. Alternatively host antibody may complex with a foreign antigen and activate complement.

After the induction of an Arthus reaction, histamine and 5-HT release peak at one hour. These agents are among those that promote increased vascular permeability, which allows a leakage of fluid and proteins into the tissues which peaks at six hours. Contemporaneous with this is the maximum cumulative influx of granulocytes. Numbers of these cells decrease over forty-eight hours. The total number of mononuclear cells is found to be maximal at twelve hours, decreasing slowly thereafter (Dunn, Giroud and Willoughby, 1976).

Polymorph Emigration

Emigration of polymorphs from the blood vessel begins after about an hour. The leucocytes become 'sticky' and adhere to the capillary wall, resistant to the blood flowing past; they put out pseudopodia and squeeze through the enlarged endothelial junctions and penetrate the basement membrane, a process known as diapedesis (Ebert and Grant, 1974; Velo and Abdullahi, 1976). The emigration probably occurs in response to chemotactic factors generated by the stimulus, for instance C5a during the activation of complement by antigen-antibody complexes. It has been demonstrated that this chemotaxin and others such as the synthetic f-methionyl peptides and a bacterial factor from *E. coli* are capable of enhancing the adhesiveness of granulocytes (O'Flaherty, Kreutzer and Ward, 1978; Smith *et al.*, 1979) so that

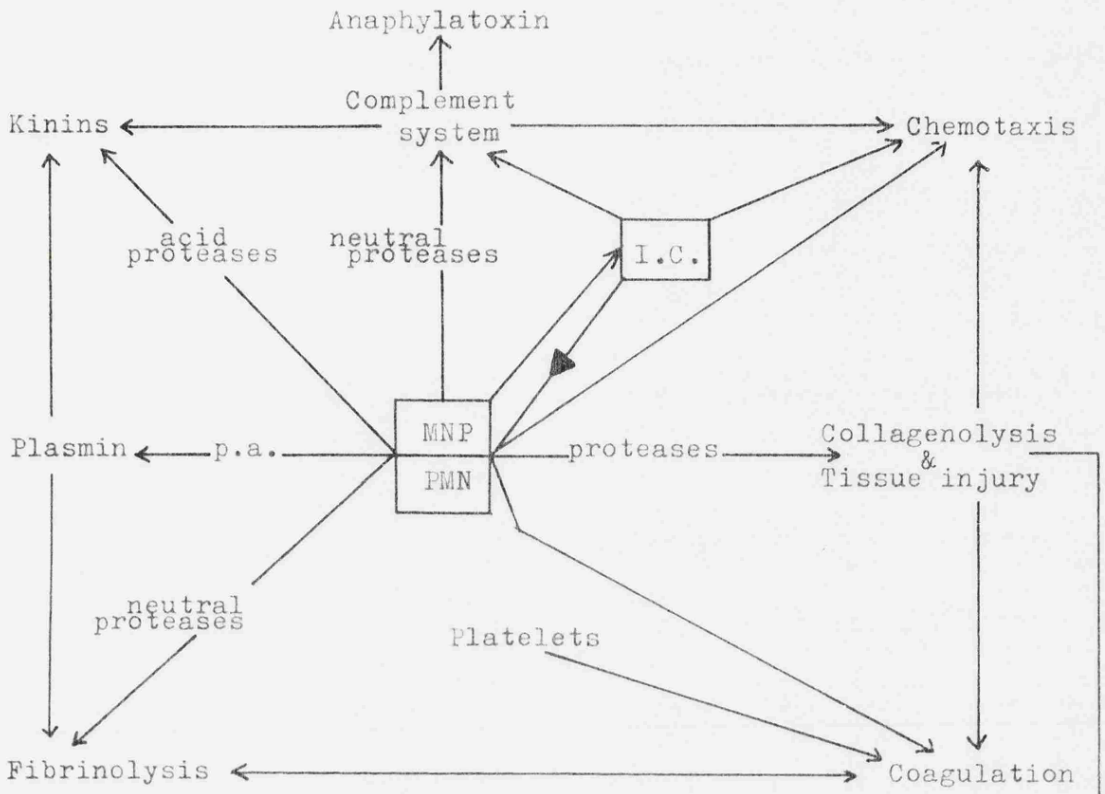
it is possible that they facilitate diapedesis, although conversely this adhesion could immobilize the cells (Fehr and Dahinden, 1979). Evidence that Clq, C3 and C4 can bind to vascular endothelium independently of antibody has been advanced recently by Linder (Linder, 1981), the tenet being that classical pathway activation of complement could occur during tissue injury without the need for the presence of antibody. This would tend to increase cellular adhesion; C5a and C3a would at the same time induce vasopermeability.

When they have crossed the capillary wall, the granulocytes migrate through the tissues towards the site of insult, where they will phagocytose anything that promotes their binding and activation. The stimulants are likely to be surface-bound and this facilitates the job of these crawling phagocytes (Henson, 1977). Rabbit peritoneal neutrophils have been shown to require certain surfaces on which to adhere before they will degranulate in response to chemotactic factors (Becker *et al.*, 1974).

As described earlier, the cells release their enzymes into phagolysosomes, and some will be discharged (through exocytosis) into the environment where they have varied actions including mediating tissue damage through proteolysis etc. Some granule constituents have been shown to increase the adherence of neutrophils and inhibit their chemotactic migration and to a lesser extent their random migration; (Bockenstedt and Goetzl, 1980); this would increase cellular accumulation. Lysozyme suppresses the chemotactic response (Becker *et al.*, 1974), but monocyte-derived lysozyme, at least, also depresses the oxidative mechanism

of neutrophils (Gordon *et al.*, 1979). The mononuclear phagocytes secrete this enzyme constitutively; since they migrate some hours after the granulocytes their product might modulate the ongoing response. The mechanism of these physiological reactions at the molecular level is incompletely understood, but membrane-protein alterations seem likely to be important in chemotaxis (Thrall *et al.*, 1980) as do ionic changes (Seligmann and Gallin, 1980) and the relative levels of cAMP and cGMP (Velo and Abdullahi, 1976). In macromolecular terms the complexities have been unravelled to a greater extent and can be pieced together in schemes such as that shown in Figure 1.4 (after Goldstein, 1974) or more simply in Figure 1.5. The inflammatory cycle could be partially tempered by the secretion of the anti-proteases α_1 antitrypsin, α_2 macroglobulin and plasmin inhibitors from the mononuclear phagocytes which follow the neutrophils, and the enzyme-inhibitor complex can be internalised and digested by macrophages (Nathan, Murray and Cohn, 1980). They also secrete the complement inhibitors C3b-inactivator and C1-inhibitor (Nathan, Murray and Cohn, 1980).

It is the events of the first few hours that are of paramount importance in the successful killing of a bacterial focus, and they directly affect the size of the lesion formed. Normally, the short-lived tissue defences act quickly, but if interrupted by, for instance, decreased local blood flow, bacteria can multiply and increase tissue damage (Leak and Burke, 1974). The time-span of induration varies with the host and the invader, but it is maximal at about twenty-four hours. There is a central area of necrosis, which results from the infiltrating neutrophils having become moribund after ingesting the bacteria.



Resulting lesion	Tissue damage to
vasculitis	venule basement membrane
arteritis	arterial elastin
glomerulonephritis	renal basement membrane
'arthritis'	cartilage
pulmonary emphysema	lung connective tissue

Figure 1.4 Inducers of inflammation.

Abbreviations used: I.C. immune complexes
MNP mononuclear phagocyte
PMN polymorphonuclear leucocyte (granulocyte)
p.a. plasminogen activator

Arrows indicate the direction of action, which may be stimulatory or inhibitory, or both.

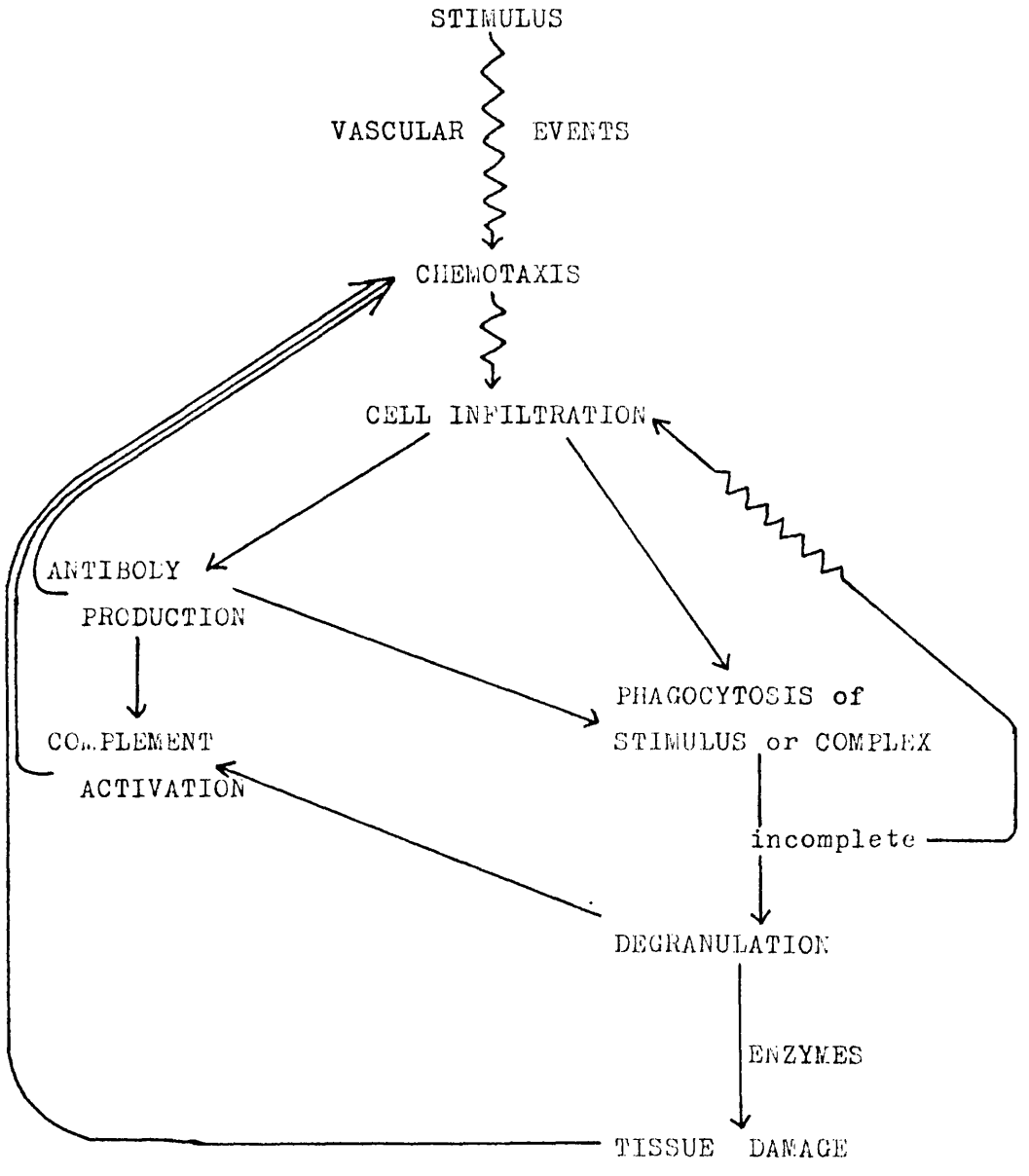


Figure 1.5

The generation of an inflammatory cycle.

~~~> represents crossing of the vascular endothelium.
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Monocytes in the tissues

The arriving monocytes by contrast are not so short-lived. They mature into macrophages with well-endowed lysosomes and a high secretory repertoire to modulate healing. They have the capacity to phagocytose both bacteria and dead granulocytes, but their ability to kill microbes is dependent upon their state of activation (Nathan, Murray and Cohn, 1980). They are able to degrade interstitial debris through the action of collagenase and elastase and fibrin by neutral proteases,to name but a few. As mentioned above they also release various enzyme inhibitors. The fate of the macrophages is poorly understood, but examination of the lymph nodes draining areas of inflammation, both acute and chronic, has suggested that many macrophages migrate to these nodes and transport dead and foreign material in their phagosomes. Moribund macrophages that die en route to the nodes may be phagocytosed by other migrating cells.

The presence of a large number of mononuclear phagocytes in the region of a stimulus probably enhances the lymphocytic response and resultant antibody production. Once coated with specific immunoglobulin the bacterium, for instance, would be more liable to be phagocytosed (Ehlenberger and Nussenweig, 1977; Henson, 1977). A continuing stimulus could be permitted through inefficient processing by either phagocytic cell type. Alternatively,the action of lysosomal enzymes in the extracellular milieu may uncover self antigens hitherto unrecognised by the host's immune system and so promote 'autoantibody'production (Fudenberg, 1978) and concomitant immune complex formation. The latter could activate

complement, induce more cellular infiltration and increase the risk of further destruction (Norman, 1974; see also Figure 1.4). The presence of local and circulating antibodies to host substances induced in this way is common, but these are not necessarily pathogenic. It has been suggested that the complexing of, for instance, anti-collagen to exposed collagen would enhance the removal of the damaged protein by macrophages (Johnson and Page Faulk, 1976; Arend and Mannik, 1979).

These examples illustrate how improper control can allow for the development of a chronic inflammatory state. The relative rarity of its occurrence after an invasive episode indicates how well managed the sequence of events is in healthy individuals. When an acute response does not give way to healing, a continuing abnormality is manifest in one or more of the contributory factors, and a chronic inflammatory state results. Chronic inflammation always passes through an acute phase.

Cells in chronic inflammation.

Of great importance to the chronic inflammatory state is the continuing presence of a cellular infiltrate, in both the initial sites and in those that have newly developed because of the deposition of immune complexes. Mononuclear cells are predominant in these long-term lesions although small numbers of polymorphs may continue to migrate for many months (Spector, Lykke and Willoughby, 1967; Vernon-Roberts, 1972). The mitotic and phagocytic behaviour of the macrophages can vary according to the nature of the lesion; the number that live for more than a few months ('long-lived') seems to be inversely proportional to the degree of cell turnover within

a granuloma (Spector, 1974). The daily entry of monocytes from peripheral blood into a Freunds Adjuvant-irritated site has been measured in the rat foot and was calculated to be 2×10^5 per 24 hours, a constant figure over the twelve weeks of study (Spector, Lykke and Willoughby, 1967). The total leucocyte influx per day into an inflamed human knee joint (in a patient suffering from rheumatoid arthritis) was calculated by Zvaifler to be 10^9 cells (Zvaifler, 1971).

A number of these mononuclear phagocytes, the proportion depending upon the type of inflammatory stimulus, transform into persistent epithelioid and multinucleate giant cells (see below). The characteristics of these cells have been studied in 'specific infective granulomata'; that is, those manifest in tuberculosis, syphilis and leprosy, which have a central zone of necrosis surrounded by these large cells (Vernon-Roberts, 1972). The epithelioid cell is an enlarged, mature form of the macrophage and can only develop if the latter has degraded all its ingested material. Low turnover granulomata, induced by for instance, carrageenan, contain macrophages that are full of undigested material; thus epithelioid cells rarely form. In contrast they are prominent in some high turnover reactions, such as that owing to B. Pertussis vaccine. The epithelioid cell is non-phagocytic, it divides into small, round cells which develop into typical macrophages which can in their turn become epithelioid cells (Spector, 1974). The multinucleate giant cell originates from the fusion of pre-existing macrophages and may contain as few as two, or in excess of fifty, nucleii, to be found at the cell periphery

(Vernon-Roberts, 1972). These giant cells take up experimentally administered carbon and thus have been shown to be present throughout the reticuloendoethelial system (now called the mononuclear phagocyte system) and at sites of tissue reaction.

The fixed tissue mononuclear phagocytes, especially the Kupffer cells of the liver, are responsible for clearing circulating complexes, although in chronic states a blockade may develop as the cells become saturated.

Immune complexes and chronic inflammation

The presence of a raised level of circulating complexes is common in animals, including humans, chronically infected with viruses of different types (Oldstone and Dixon, 1971) Such complexes are associated with glomerulonephritis and/or arteritis (Leber and McCluskey, 1974). Soluble immune complexes of less than 19s pass through, rather than deposit at, the glomerular basement membrane: large lattices are phagocytosed by the Kupffer cells: so it is the intermediate sizes that deposit (Cochrane, 1971). Most of these bind complement and this can be demonstrated with the use of fluorescein-labelled anti-C3 which follows closely the pattern found with fluoresceinated anti-immunoglobulin in the same glomerulus (Cochrane, 1971; Oldstone and Dixon, 1971).
This tissue deposition and complement activation reinforces the inflammatory impetus by causing local tissue damage with the consequent chance of autoantibody formation. A likely consequence of the interaction of immune complexes and complement is hypocomplementaemia; in terms of the ongoing reaction this state might be

considered advantageous in regulating the response, but the ability of the host to opsonize infective bacteria would also be impaired.

Autoantibodies do develop in infections, especially in prolonged ones with specific infective granulomata, in which the host is ineffective in combating the disease. This auto-antibody formation is the case, for example, in patients suffering from sub-acute bacterial endocarditis, where the specificity of the anti-bodies range from anti-IgG ('rheumatoid factor ') and antinuclear factor to anti-thyroid cell antibody. Antibodies to Ubiquitous exogenous antigens such as Candida have also been found to be common despite an apparent absence of, for instance, this organism. The incidence of the auto-antibodies paralleled more severe and persistent disease and decreased markedly with recovery of the patient. (Bacon, Davidson and Smith, 1974). Circulating complexes were found in all patients with definite infective endocarditis by Burton-Kee and workers (Burton-Kee, Morgan-Capner and Mowbray, 1980), although the presence of antigen in these complexes could not be demonstrated. They too noticed the presence of 'rheumatoid factor' in the serum which correlated with complement binding IgG and IgA complexes. During the time that circulating immune complexes are produced during an acute infection, such as in typhoid fever, they too are accompanied by this anti-immunoglobulin G activity Rajagopalan, Kumar and Malaviya, 1981).

Rheumatoid factor and its specificity

Rheumatoid factor (RF) derives its name from the disease in

which it is most commonly found, that is, in rheumatoid arthritis. It is present in the serum of between 60 and 80% of patients with this disease, where it persists, often at high titre, in contrast to the transient nature of its appearance during acute infection. Johnson and Page Faulk (1976) have compiled a comprehensive review of its properties, knowledge of which has accumulated since the 1940's when rheumatoid sera were seen to be able to agglutinate sheep erythrocytes sensitized with rabbit antibody. This publication has therefore been extensively drawn upon in the following section.

The RFs that have the ability to agglutinate IgG-coated latex particles and red cells, and to precipitate soluble aggregates of IgG, are IgM antibodies directed against antigenic moieties on the Fc portion of human IgG, although there is a cross-reaction with rabbit IgG. This IgM RF is almost always 19s (pentameric), the ten possible valencies being reduced to five in practical terms because of steric hindrance. The monomeric subunit, low molecular weight IgM, can be demonstrated by radio-immunoassay (RIA) techniques, and is implicated in the disease process (Roberts-Thompson, Wernick and Ziff, 1981). IgG RF activity is poorly detected using agglutination techniques but more sensitive RIA have shown it to be present in the sera and synovial fluid from rheumatoid patients both seropositive (i.e. positive for IgM RF) and seronegative (Shakib and Stanworth, 1978; Allen *et al.*, 1981).

Both 19s (IgM) and 7s (IgG) antiglobulins in sera from rheumatoid arthritis patients have been found to be enriched for K-light chain containing antibodies in comparison to the ratio of kappa to lambda in each individual's globulins (19s + 7s) (Carson and Lawrance, 1978). Patients with sub-acute bacterial endocarditis show a similar change in the 19s antiglobulins, but not in the 7s. This predominance has been demonstrated, experimentally, to occur as a result of repeated stimulation with antigen, and the above authors suggest that the presence of kappa-chains may represent an idiotypic marker related to rheumatoid factor production.

The study of the specificity of RF has largely been confined to that of the IgM-antiglobulin, and has followed three general directions: determinants on native IgG; on complexed but not native IgG; and on hidden sites only exposed following enzymic treatment.

In the first case, the genetically-determined immunoglobulin allotypes looked interesting. In accordance with the WHO recommendation (World Health Organization, 1976), the terminology for these is Gm (γ-chain markers), Km (κ-chain markers) and Am (α-chain markers) with a number denoting the subclass on which the are found e.g. G1m, A2m. The Gm markers have been the most comprehensively studied and at least 22 are known and identifiable. In normal sera the anti-Gm activity is usually of the IgM class (Divall and Khalap, 1976) and reacts only with the IgG that carries the antigenic marker for which it has specificity. The subject himself is not positive for that determinant. In contrast,

IgM RF is not specific in this way though it may act as an anti-Gm agglutinin. It can be inhibited not only by the appropriate Gm positive serum but also by the negative, and it can also react with autologous IgG. The allotypes are mutations at single, or a narrow range of, amino-acids in the polypeptide chain and their antithetic markers are therefore widely distributed amongst the immunoglobulins. It is these that could well be implicated in the reactivity of RF (Gaarder and Natvig, 1970; idem, 1972), but if so they seem to occur concurrently with activity towards a non-allotypic determinant called Ga (Gaarder and Natvig, 1970; Gaarder and Michaelsen, 1974) which was originally detected by Allen and Kunkel (1966) in a rheumatoid donor. This patient's antibody circulated complexed to her own IgG and was dissociated on an acidic gel-filtration column. The Ga antigen is present on IgG1, 2 and 4, but not IgG3;& interestingly the reaction of IgM RF and rabbit IgG too has been found to be inhibited by all subclasses but IgG3 (Gaarder and Michaelsen, 1974). The Fc region of IgG inhibits the reaction between anti-Ga and its substrate (Allen and Kunkel, 1966). It has been suggested that RFs interact with antigens located on both homology regions of the Fc fragment, one on the Cγ3 and three on Cγ2 (Natvig, Turner and Gaarder, 1972).

There is some controversy as to whether RF can bind to native as well as to complexed or aggregated IgG. Certainly it binds far better to the latter, which suggests that new antigens may appear as a result of the conformational chnages that have occurred. The finding that circulating RF is found complexed

with monomeric 7s IgG as in the case of the patient Ga, above, suggests that the IgG may not be native but instead may already be bound to an as yet undefined antigen. Alternatively, the interaction may be more efficient solely because of the increased local concentration of the IgG molecules.

The sera and joint fluids of patients with rheumatoid arthritis often contain antibodies with activity towards areas uncovered by pepsin digestion of normal IgG. These so-called 'pepsin agglutinators' are also found in some chronic infections and in normal sera. They show a specific reactivity towards the $F(ab')_2$ fragment, which is similar to that produced by lysosomal protease action. This hints at a usefulness in clearing IgG that has been degraded during the inflammatory response in much the same way that anti-collagen antibodies are produced to remove damaged fibres. The cross-reactivity of rheumatoid factor activity and the presence of other autoantibodies in rheumatoid arthritis are discussed in a following section.

Rheumatoid factor and complement fixation.

Monoclonal IgM RFs manifest a variation in complement-fixing ability but those in heterogenous polyclonal serum can do so when bound to complexed IgG. The interaction between RF and IgG-coated latex particles has been shown to be modified by the presence of complement in fresh serum, or isolated C1 component (Hallgren, 1980). This factor is thought to bind avidly to the 'complexed' IgG and decrease the ability of the RF to also bind. If, however, the complement is artificially activated, the interactions are not inhibited.

IgG from plasma only poorly energizes complement, if at all (Pope, Teller and Mannik, 1975), but if bound to IgM RF the stabilized form may allow the latter to act upon complement (Allen *et al.*, 1981). Winchester, Agnello and Kunkel (1971) characterized the IgG-IgG complexes that are found in the serum and the joints of rheumatoid arthritis patients and interestingly discovered that those in serum did not bind complement while those in the synovial fluid did so readily (Winchester, Kunkel and Agnello, 1971; Winchester, 1975).

The pepsin agglutinators discussed above exhibit negligible complement fixation.

Rheumatoid factor and its relevance to rheumatoid inflammation.

The presence of IgM RF in serum seems to have some prognostic implications for the patient, with high levels being associated with more severe disease and systemic manifestations such as cutaneous vasculitis and nodules. Recently a strong correlation between IgG RF and vasculitis has been noted (Allen *et al.*, 1981; Scott D. *et al.*, 1981). The patients in this study (Scott D.G.I. *et al.*, 1981) were found to have circulating complexes with anti-complementary activity, and serum C4 levels below the normal range, which indicated that the IgG RF-containing complexes bound complement. IgM RF was also present in the sera.

Self association between IgG RFs was noted by Pope, Teller and Mannik (1975) and IgM/IgG by Jones and colleagues (1980); this may generate intermediate-sized complexes which could account

for the plasma hyperviscosity that tends to be found in patients with rheumatoid arthritis.

Whether an IgG molecule is bound to a small antigen or to another IgG molecule, the addition of an anti-immunoglobulin will increase the size of the complexes and possibly their tissue deposition (Cochrane, 1971) and promote their removal by scavenging granulocytes or monocytes, or by the Kupffer cells. This deposition could promote the risk of incomplete phagosome formation with the possible consequences that have been discussed in a previous section. On the other hand, IgM RF might decrease their uptake by neutrophils since these cells do not have IgM receptors (Ehlenberger and Nussenweig, 1977).

The effect of both IgG and IgM RFs on the formation and processing of DNA/anti-DNA complexes has been studied by Lamers (1980 and 1981). He discovered that the effect of the IgM RF was very dependent upon the size of the complexes and the presence of complement, whereas IgG RF increased the size and processing of small complexes but had virtually no effect on large ones. Alkner and Hansson (1979) have noted that IgM RF affects the equilibrium of antigen-antibody reactions.

Since the major feature of rheumatoid disease is arthritis, the presence in the synovium and synovial fluid of rheumatoid factor has been investigated. The technique of immunofluorescence has been used to demonstrate the presence of IgG, IgM and complement in a wide range of synovial tissues from rheumatoid

patients, but this provides circumstantial evidence only for the presence of complexes. Plasma cells are concentrated in inflamed synovium and appear to contain RF activity. This can, however, only be successfully demonstrated after a pepsin digest of the tissue has been made, which has been interpreted to mean that self-association of IgG RF has already occurred (Natvig and Munthe, 1975).

The possibility that RFs are only secondary to the disease process has been reinforced by some evidence. Anti-immunoglobulins are present in diseases other than rheumatoid arthritis, as discussed previously, and only decrease as the patient recovers. Patients with hypogammaglobulinaemia can suffer from a rheumatoid-like arthritis without producing any RFs; and only a few youngsters with juvenile rheumatoid arthritis (JRA) develop rheumatoid factor.

It might be helpful to type the specificity of the anti-antibody activity from infectious diseases and make a comparison with the anti-Ga activity of rheumatoid sera.

Linked to the pathogenesis of these factors is the stimulus for their production, and this is as yet unknown. There are a number of theories, some of which are outlined below.

A popular theory at present is one in which the immunoregulatory system is aberrant, a state possibly induced by a chronic viral infection (Depper and Zvaifler, 1981; Fudenberg, 1980; Panayi,

Corrigall and Youlten, 1981). The Epstein-Barr virus (EBV) can cause a number of diseases in which alterations of the immune system occur, for instance infectious mononucleosis (which is accompanied by autoantibody production) and Burkitt's lymphoma (Depper and Zvaifler, 1981). When cultured mononuclear cells are infected with EBV, they can transform into permanent cell lines, a process followed by rising levels of IgM in the culture supernatant. When the cells are derived from rheumatoids, in comparison to normals, the amount and affinity of the IgM anti-IgG produced is higher in the former group, though the total IgM remains similar (Slaughter *et al.*, 1978). Normal human lymphocytes can likewise be stimulated by pokeweed mitogen to secrete IgM RF *in vitro* (Koopman and Schrohenloher, 1980). Lymphocytes apparently uninfected with EBV had higher transformation scores in RA patients (6 of 11) compared to normal controls (2 of 10), suggesting persistence of the virus within the mononuclear cells. The work of Alspaugh and Tan (1976) and Alspaugh *et al.* (1978) has also indicated that this is the case, because about two-thirds of rheumatoid sera (sero-positive) contain an antibody to a nuclear antigen present in human lymphocyte cell lines, and which is expressed when such cells have been transformed specifically by EBV. This was termed RANA (rheumatoid arthritis-associated nuclear antigen) to distinguish it from the Epstein-Barr nuclear antigen, EBNA. EBNA is expressed only transiently after the cell is infected; by contrast RANA is permanent. Only eight percent of normal sera have such antibodies. About eighty percent of the world's population have been infected with EBV, usually asymptomatically, so that if it is to be implicated as a causative agent in rheumatoid arthritis, environmental and personal factors

need to be considered (Panayi, Corrigall and Youlten, 1981).

A caveat to the study of antibody activity such as that described above is that rheumatoid factor shows a marked degree of heterogeneity in the antigens with which it can bind. For instance, Johnson has extensively studied its expression of an anti-nuclear activity unrelated to RANA (Johnson, 1979, 1980) and Bokisch and colleagues (1973) have reported a strong cross-reactivity towards peptidoglycan in a rabbit 7s anti-IgG that was produced during immunization to streptococci.

A very different idea to that of abnormal immunoregulation is that RF production is stimulated solely because the patient's IgG has become immunogenic, either by complexing (with antigen or another immunoglobulin), aggregation or enzymatic digestion. This may be the stimulus for its production in acute illness as mentioned previously. The antigenicity generated might well depend upon the nature of the antigen that was bound. Related to this is the concept that the patient's IgG is inherently structurally altered. Circular dichroism (CD) has been used to study the conformation of immunoglobulins. In comparison to a normal reference, immunoglobulin G from RA patients has been shown to have altered CD spectra around two wavelengths, namely 280 nm and 294 nm, which reflect hinge region (certain disulphide bonds) and tryptophan resonance respectively (Johnson *et al.*, 1974). The differences are probably attributable to IgG anti-globulins or the IgG to which they are attached. This idea has recently been strengthened by Alkner and co-workers (1982) who have shown that IgG complexes with

RF activity, isolated by affinity chromatography, show similar changes while the patient's monomeric IgG has behaved normally. This is the case under dissociating as well as neutral conditions which indicates an intrinsic, rather than antigen-binding, aetiology. The deviant conformation at the hinge region is interesting because this area can influence binding to membrane Fc receptors, and thus indirectly to B-lymphocyte responsiveness (Johnson *et al.*, 1975; Johnson, Watkins and Holborow, 1975).

There is evidence from the Gm and Km systems that materno-foetal incompatibility can result in either mother or baby, or both, being actively immunized by the other for the non-shared allotypes. Investigation did not implicate these in JRA at least,, It has been of interest because only IgG crosses the placenta, and RF reacts solely with IgG amongst the immunoglobulins. It has been stressed that the presence of rheumatoid factor is a characteristic of some infective processes, but that it tends to be transient. The disease in which it was first found, and in which it is most commonly, and continually, present is rheumatoid arthritis. No infective organism, in whole or part, has, as yet, been shown to be the causative agent in this chronic inflammatory disease (e.g. Wilkes *et al.*, 1973). The fact that the persistance of a virus within a joint can cause a long-term arthritis has recently been illustrated in a study of five patients who failed to show clinical symptoms of Rubella infection (Grahame *et al.*, 1981). Despite this, a cytopathic agent indistinguishable from rubella virus was demonstrated in their synovial fluids and all patients had high haemagglutination titres to rubella. A transient arthritis

concurrent with a clinically evident rubella infection is not uncommon. Interestingly, like RA (Masi and Medsger, 1979), it occurs predominantly in females (Grahame *et al.*, 1981). The difference between this major group (transient symptoms) and the minority of patients with chronic synovitis (discussed above) stresses the importance of the host's immune system in handling foreign agents, a factor discussed in the previous section.

Gastrointestinal infections with organisms such as Yersinia, Shigella or Salmonella can also be followed by a reactive arthritis. Individuals who are susceptible to this tend to be HLA-B27 positive (Laitinen and Leirisalo, 1981). This reinforces the suggestion that there is genetic control over the inflammatory and immune states.

Rheumatoid arthritis; pathogenesis.

Rheumatoid arthritis is a disease in which the control of these processes seems to be aberrant, and which is probably multifactorial in origin (Panayi, Corrigall and Youlten, 1981). It is primarily a disease of joints, and is first manifest as inflammation of the synovium, which then progresses to a proliferative synovitis which results in damage to bone, cartilage and tendons. The early synovitis is characterized by an increase in blood flow to the synovium; a migration of granulocytes from the circulation into the joint space, and of mononuclear cells into the perivascular regions of the tissues. The predominance of polymorphonuclear leucocytes within the fluid persists even in the chronic state, when they form about 70% of the 19 million leucocytes per ml., on

average, found in the rheumatoid joint (Cohen and Skinner, 1979). The pro-inflammatory nature of leucocyte secretions has been discussed in a previous section, and may explain the establishment of the chronic inflammatory state. The abnormal highly vascular and cellular synovium interacts with connective tissue cells to form a pannus that progresses from the joint periphery towards its centre, eroding cartilage and bone along its path (Harris, 1979). Histological and electron microscopic studies of the pannus-cartilage junction have identified granulocytes (Mohr, Westerhellweg and Wessinghage, 1981) as well as mononuclear and synovial cells, and the tissue destruction is likely to be mediated by enzymes from several cell types (Harris, 1976). The inhibitory proteins α_2-macroglobulin and α_1-anti-trypsin may become saturated, both locally and in the synovial fluid, and thus unable to regulate enzyme activity.

The processes of erosion and proliferation alter the architecture of the joint such that it is less moveable and may undergo partial subluxation (Gardner, 1972): but it also uncovers potentially antigenic moieties through the action of the secreted enzymes. Proteolgycan and collagen are both damaged by the progressive degradation (Lancet, 1977). Glant and co-workers have used an ^{125}I-radioimmunoassay (solid phase) to detect anti-proteoglycan antibodies in the sera of eleven out of twenty-nine patients with RA, and in 4 of 6 rheumatoid synovial fluids (Glant, Csongor and Szues, 1980). Neither normals nor ankylosing spondilitis patients' sera reacted to the antigen, proteoglycan of human articular cartilage. Leucocyte migration inhibition and lymphocyte

stimulation, that is cell-mediated immunity, were also demonstrated during the same study.

Menzel (Menzel *et al.*, 1978) utilized a ^{14}C-radioimmunoassay to demonstrate the presence of antibodies to denatured type I human collagen in RA synovial fluids; 20/27 (74%) were positive, sixteen of which also gave a titre of greater than 1 in 16 by passive haemagglutination. Antibodies to native collagen were found in only eight of the twenty-seven, and inhibition experiments suggested that this activity was because of cross-reacting anti-denatured-collagen antibodies. Similar inhibitory tests indicated that there was also cross-reactivity towards type III denatured collagen. The use of immunofluorescence has detected anti-collagen antibodies in just 3% of patients, most of which were highly specific towards type II collagen (Beard *et al.*, 1980). The disparate frequency of these results may be partly explained by the different techniques used, immunofluorescence being less sensitive than radioimmunoassay.

In agreement with Glant's work with proteoglycans, Stuart *et al.* (1980) have found that peripheral blood MNC from patients with joint diseases have been shown to possess cell-mediated immunity towards native collagens and their α-chains. The immune activity was more frequent and intense with RA patients than, for instance, ankylosing spondilitis victims or normals. For native collagens this was 50, 20 and zero percent respectively, and towards denatured α-chains 90, 50 and 10 percent.

Some cross-reactivity between human Clq and human collagen

has been demonstrated in a delayed-type hypersensitivity reaction in guinea pigs (Menzel, Smolen and Reid, 1981). It is conceivable that the presence of complement-binding immune complexes might therefore enhance this cellular activity.

The presence of collagen/anti-collagen immune complexes has been indicated in synovial fluid cells from RA patients (Steffen, Ludwig and Knapp, 1974).

Immunoglobulin is secreted by rheumatoid synovial membrane (Smiley, Sachs and Ziff, 1968) and may itself by damaged by proteolytic action and so become immunogenic. But as an antibody it could bind to its antigen (either the causative agent or host neo-antigens) and be altered by virtue of its being complexed. This change has been discussed in the previous section.

The arthritic joint exhibits many of the features found in chronic inflammatory states and which were outlined in Figure 1.4. For instance, leucocytes are induced to migrate from the capillaries (chemotaxis). The same vessels become more permeable and allow seeping of macromolecules such as clotting factors and fibrinogen into the environment; fibrin is deposited on, and in, the synovium (Harris, 1979). These examples, whatever their cause, probably provide enough impulsion to start the wheel of inflammation revolving.

Synovial tissue and fluid.

The native synovial cells may themselves be important in this cycle. There are basically two major types of synovial cell;

Type A (macrophage-like) and Type B (fibroblast-like). The former have surface villi and sparse endoplasmic reticulum (ER), and their lysosomes become more abundant in RA, where residual bodies are also found in the cytoplasm (Gardner, 1972): both indicate active phagocytosis. The Type B cells possess abundant ER which is probably involved in making glycoprotein for export. Barratt and colleagues (1977) have made a study of pig synovium and have concluded that the synoviocytes of the superficial layer belong to one cell type. They assume either the A- or the B- type according to local environment conditions.

Synovial fibroblasts secrete hyaluronic acid, and rheumatoid-derived cells, *in vitro*, have been shown to secrete a hyaluronate that is less viscous than normal. Since the normal, high molecular weight compound has anti-inflammatory effects (Harris,1979), the altered species may have a profound effect on the ongoing cellular response.

Giant multinucleate cells are also found in the synovium and tend to be associated with the presence of IgM RF in the plasma (Gardner, 1972). They are probably of macrophage origin (Harris, 1979) as has been found at tissue reaction sites in other diseases (Vernon-Roberts, 1972).

Structural features of the joint have a bearing on the disease process for the loose organization of cells in the synovium allows its proliferation in arthritis. The encapsulated nature of the joint means that there can be an influx of viable granulocytes

towards a stimulus, but limited exit for the moribund cells, which must therefore be degraded *in situ*. The vast increase in cell numbers, although concurrent with an increased blood flow, leads to a decrease in the partial pressure ofoxygen and of pH as the pCO_2 and lactate levels rise (Harris, 1979). Many of the lysosomal enzymes that enter the fluid by exocytosis are acid hydrolases which degrade their substrates more efficiently at acidic pH. This may facilitate the damage to cartilage that occurs independently of the pannus.

Immune complexes have been shown to be present in synovial fluid, and sera, from RA patients (Winchester, Kunkel and Agnello, 1971) and are thought to be responsible for perpetuating the inflammatory response (Male, Roitt and Hay, 1980). They certainly have the ability to initiate and maintain extra-articular reactions; vasculitis is associated with high levels of IgG RF and raised immune complexes (Allen *et al.*, 1981; Scott D.G.I. *et al.*, 1981) as has been found in animal models (Leber and McCluskey, 1974). The subcutaneous and pulmonary nodules that are found in some RA patients are also vasculitic in origin (Harris, 1979; McGuire and Harris, 1979). The measurement and analysis of complexes with respect to RA has, therefore, been popular (e.g. Onyewotu *et al.*, 1975; Erhardt, Mumford and Maini, 1979; Male, Roitt and Hay, 1980). However, there is a lack of correlation between the many different assays available because they are specific for different properties, and this has made the interpretation of data difficult (Soothill, 1977).

The presence of activated C4 in synovial fluid containing complexes indicates strongly that the latter are major activators of complement (Male, Roitt and Hay, 1980) in this specific case as well as in general (Figure 1.4).

Regulation

The multiplicity of possible regulatory sites in the inflammatory process makes the study of chronic inflammatory diseases very complicated. Given that rheumatoid arthritis is of unknown etiology, it is valid to ask if there is some cellular or humoral defect in immunoregulation, either in isolation from, or related to, an infectious agent (Fudenberg, 1980; Panayi, Corrigall and Youlten, 1981). Specific investigations of leucocytes, and in particular phagocytes, will be discussed in subsequent chapters. The properties of the granulocytes would seem to be particularly important since they are the first cells to reach a pro-inflammatory site, and to ingest and degrade an offending organism. The closely following mononuclear phagocytes are much longer living and are thus very important in chronic lesions. It was therefore decided to study some of the properties that these cell types manifest, in particular those related to their interaction with complexed immunoglobulin including rheumatoid factor. The phagocytes from rheumatoid arthritis patients and healthy controls were to be compared in these respects in order to ascertain if any differences existed.

CHAPTER 2. GENERAL METHODS

1. Preparation of cells:

The anti-coagulant used in most instances was citrate, 1.1 millilitres (mls) of 0.106 M trisodium citrate being used for 10 mls of peripheral blood. For some experiments, preservative-free heparin was used at 125 U per 10 mls of blood.

The leucocyte populations were separated from one another using either Percoll or Ficoll-Paque (Pharmacia Fine Chemicals), according to the methods outlined in the following chapter. Where red cells contaminated the leucocyte preparations, they were removed by differential lysis using an ammonium chloride solution of the following composition:

155 mM NH_4Cl
10 mM $KHCO_3$
0.1 mM EDTA

This has a final pH of 7.3 - 7.4 (Weening, Roos and Loos, 1974). One ten minute treatment with the above was usually sufficient to remove the unwanted cells. The leucocytes were washed a further twice (whether or not they had undergone this treatment), in an iso-osmotic PBS containing 0.2% w/v egg albumin (Sigma), termed PBSA. They were then resuspended in PBS or in tissue culture Medium 199 containing 20 mM HEPES and 10% heat-inactivated foetal calf serum (FCS) (Wellcome and Gibco-Biocult).

2. Evaluation of cell preparations:

All cell counts were carried out in white cell counting fluid containing 3% acetic acid and 0.1% methylene blue (British

Drug Houses) and all cell viabilities in trypan blue (0.2 g dl^{-1}) in PBS, using a Neubauer counting chamber.

A cytocentrifuge (Shandon Southern, Runcorn) was used to make the cytological preparations. Approximately 2 x 10^5 cells were resuspended in 3 mls of cold PBSA and spun onto four slides at 500 r.p.m. for ten minutes. The slides were air-dried, fixed in methanol and stained for ten and twenty seconds in the eosin and haematoxylin-based stains respectively, of Diff-Quick (American Hospital Supplies Ltd.). Each stained preparation, including blood smears fixed and stained as above was mounted under a cover-slip with DPX (Raymond Lamb Ltd.).

3. Phagocytosis assays:

The stimulus that was used most commonly was human IgG that had been prepared from an IgG1 myeloma serum (see section 2.5(iii)). It was aggregated at 60°C for 30 minutes at a protein concentration of 4.25 mg ml^{-1} (section 2.5(vii)). This aggregate was particulate and had an anti-complementary activity (ACA, performed by Immunology Department, Royal United Hospital, Bath) of greater than 1 in 4096. When the aggregate was centrifuged at 5000 g for 30 minutes and the supernatant subject to gel filtration on Sepharose 6B-Cl (Pharmacia), there was no evidence of IgG being present.

Another IgG1 myeloma, when purified, reacted to heat differently and produced a soluble, not a particulate aggregate with an ACA of 1 in 32. This was referred to as soluble aggregate (see Section 2.5 (vii)).

The assays were set up in polystyrene tubes (Luckams Ltd., Sussex), with neutrophils at a final concentration of 5×10^6 ml^{-1} and monocytes at 1.25×10^6 ml^{-1} in M 199 plus 10% FCS, with or without the stimulus at the desired concentration. The stoppered tubes were incubated at $37^{o}C$ for one hour on a rocking table (4RT/6-10,Luckams). Subsequent to this, they were spun at 1000 g for 15 minutes at a temperature of $4^{o}C$, and the supernatants harvested. For lysates, each cell pellet was made up to the original incubation volume with a solution of 0.1% bovine serum albumin (BSA) with 0.1% Triton-X-100 in saline. This was well mixed and then centrifuged at 5000 g for 25 minutes. The supernatants were harvested. The test supernatants and lysates were used fresh, or stored for up to two weeks at $-20^{o}C$, for the estimation of the lysosomal enzymes . The estimation of lactic dehydrogenase (LDH) was always carried out on fresh samples.

4. Enzyme assays:

Four different lysosomal enzymes were assayed, and the details are laid out in Table 2.1. All the substrates and standards were purchased from Sigma (Poole, Dorset) and other chemicals from BDH (through Ferris, Bristol). When the levels of the β-glucuronidase and β-galactosidase were measured in cell lysates, the sample volume that was added was halved, and the corresponding values doubled. All four enzymes were measured by endpoint reactions, while the lactic dehydrogenase was estimated using a kinetic reaction.

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ENZYME	SUBSTRATE	BUFFER	SAMPLE VOLUME	TIME AND TEMPERATURE	STOPPED WITH	λnm
β-glucuronidase	50 µl of 0.1M	350 µl 0.1M acetate pH 4.5	100 µl	22 hrs, 37°C	2 mls 0.4M glycine/NaOH	420
β-galactosidase	50 µl of 0.015M	350 µl 0.3M citrate pH 5.0	100 µl	22 hrs, 37°C	2 mls 0.4M glycine/NaOH	420
acid phosphatase	10 µl of 79 mg ml^{-1}	650 µl 0.12M citrate pH 4.8	100 µl	10 mins, 37°C add substrate, 1 hr, 37°C	50 µl 4N NaOH	405
peroxidase	24.4 mg in 1 ml of 1M HCl and 9 mls EtOH	3.2 mls in 100 mls 0.1M citrate pH 5.5 containing 10 µl 30% H_2O_2 and 0.05% Triton X 1 ml volume	50 µl	5 mins, 37°C	1 ml 35% perchloric acid	560
lactic dehydrogenase	20 µl of 5 mg in 640 µl Tris /HCl	1.2 mls of 0.63mM pyruvate in 0.05 M Tris/HCl pH 7.5	100 µl	Δ1 min, 25°C	-	340

STANDARDS		SUBSTRATES
β-glucuronidase	EC 3.2.1.31	p-N-phenyl-β-D-glucuronide pyranoside
β-galactosidase	EC 3.2.1.23	p-N-phenyl-galactopyranoside
acid phosphatase	EC 3.1.3.2	p-N-phenyl-disodium orthophosphate (Sigma substrate 104)
horseradish peroxidase	EC 1.11.17	o-dianisidine
lactic dehydrogenase	EC 1.1.1.27 (Type XII, from human erythrocytes)	nicotinamide adenosine dinucleotide (reduced form)

(All Sigma reagents)

Table 2.1. The assessment of enzymes from phagocytes. Methods for assaying the acid hydrolases and the cytoplasmic enzyme lactic dehydrogenase were kindly supplied by Boots Ltd., Nottingham. The acid phosphatase method here was modified slightly from that supplied. The peroxidase was assessed by the method of Bretz and Baggiolini (1974) as used by Segal, Dorling and Coade (1980).

5. Preparation of protein solutions:

(i) Chromatography and Estimation

All the chromatographic reagents were purchased from Pharmacia Fine Chemicals, whose columns were used in most procedures. Comparable apparatus manufactured by Wright Scientific Ltd. was also used. Fractions were collected and recorded on an LKB 7000 fraction collector and chart recorder. Protein estimations were carried out by the method of Lowry *et al.* (1951). Where the size of the sample precluded this, the concentration was calculated from its optical density at 280 nm, according to the following equation:

$$Abs_{280} \times 0.72 = \text{concentration (mg ml}^{-1}\text{)}$$

(for immunoglobulins)

(ii) Purity of the preparations.

The immunodiffusion was carried out in 1% agar made up in 0.05 M Tris HCl pH 8.6 containing 0.1% sodium azide. The immunoelectrophoresis was carried out on glass photographic plates coated with 1.5% agar in barbitone buffer in a bath filled with sodium barbitone/acetate buffer pH 8.6. It was run at approximately 40 mA, 200 V for 2.5 hours with bromophenol blue as a migration indicator. Shandon Southern equipment was used. Once this stage had been completed, antiserum was added to the troughs and an incubation carried out at room temperature overnight in a moist chamber. After drying, the plates were stained for 30 minutes with 0.1% Ponceau S in 3% trichloroacetic acid, and destained in 5% acetic acid followed by fixing to the plates in 2% glycerol for 2 hours. The

immunodiffusion plates were also stained in this way following extensive washing in saline. This staining method, and that of the IgG preparation (see below) are routinely used at the Scottish Antibody Production Unit, at Law Hospital, Lanarkshire (SAPU).

Antisera were purchased from Boehring, except for the anti-human IgG γ-chain specific which was kindly donated by Dr. A. Munro of SAPU.

(iii) IgG was purified from human myeloma serum as follows:

A DEAE-Sephadex column (1.5 x 30 cm) was prepared equilibrated in 0.02 M phosphate buffer, pH 6.2. Five mls of the patient's serum was dialysed against the same buffer for 24 hours at 4°C and applied to the column. The IgG was eluted in the same buffer and was dialysed back to neutrality against PBS, in a positive pressure Amicon unit with a PM 50 membrane. All other proteins were eluted from the column with 2 M NaCl in 0.02 M phosphate, and discarded. The IgG was pure by immuno-diffusion (Ouchterlony) and by immuno-electrophoresis.

(iv) Fractionation of synovial fluid:

The following method was used to isolate putative immune complexes from rheumatoid synovial fluid.

The euglobulin fraction was precipitated with 45% ammonium sulphate and allowed to flocculate overnight at 4°C before being washed twice with 45 % ammonium sulphate. The precipitate was dissolved in 20 mls of 0.02 M phosphate buffer, pH 6.2 and dialysed for 36 hours against the same buffer before being passed over

DEAE-Sephadex as described above (Section 5 (iii)). Complexes could not be further separated from the bulk of the IgG using a Sepharose Cl-6B column (Wright) in PBS, pH 7.4. The buffer contained 10 mM phosphate and 150 mM NaCl. The column used was 1.6 x 90 cm and was run at 8 mls per hour with a positive peristaltic pump (LKB).

The IgG peak was aliquoted and stored at -90°C, at 2.9 mg ml^{-1}. This preparation had an ACA of 1 in 512, which decreased slightly to 1 in 128 after eighteen months storage.

(v) Rheumatoid factors:

IgM rheumatoid factor, purified by the Degalan Column technique (Johnson, 1979) was kindly provided by Dr. G. Onyemelukwe (University of Liverpool). The mixed IgM plus IgG rheumatoid factor was donated by Dr. C. Elson (University of Bristol), who prepared it from the serum of a patient attending the Rheumatic Diseases Hospital in Bath.

(vi) Preparation of immunoglobulin allotype reagents:

The IgM fraction of an anti-Glm (f) serum was prepared by dialysis of the serum against distilled water for 48 hours. The resulting precipitate was centrifuged at 2,200 g for 20 minutes and the supernatant discarded. The pellet was resuspended in 10 mM phosphate/150 mM NaCL pH 7.4 and allowed to dissolve overnight at 4°C.

Small aliquots of about three milligrams each were applied

to a Sepharose Cl-6B column, 1.6 x 90 cm in the same buffer, run at 8 mls per hour, as above. The first peak and its shoulder were pooled and concentrated in an Amicon unit to 2.5 mg ml^{-1}. This contained three components by immunoelectrophoresis: IgM, α_2 macroglobulin and lipoprotein. One percent bovine serum albumin was then added to stabilize the IgM solution.

The activity of both antiserum and IgM fractions towards Glm (f) positive IgG was evaluated by a standard haemagglutination assay. This involved sensitizing a pool of Group O positive red cells with an anti-D positive for Glm (f). This was carried out at 37°C for 1 hour after washing of the red cells in PBS, and was followed by further extensive washing to remove unreacted anti-D.

The antiserum or IgM fraction was allowed to react with them at room temperature for one hour in small polystyrene tubes. After a short centrifugation, 35 g for 3 minutes, the cells were carefully streaked onto a grease-free glass plate and studied for the presence of agglutination.
IgG from a Glm (f) positive serum was prepared using the same method as that used for the myeloma IgG.

All the IgG allotype reagents were kindly provided by Mr. P. Holt of the Blood Transfusion Service at Southmead Hospital, Bristol.

(vii) Aggregation of IgG:

The IgG1 myeloma (Mrs H) with which most of the

experimental work was carried out was found to aggregate and precipitate out very easily at 60°C or above. Samples of the purified IgG at 4.25 mg ml^{-1} in PBS were incubated at 60°C for increasing periods of time, plunged into crushed ice, then centrifuged at 2000 g for 20 minutes. The protein concentration of each supernatant was measured and the amount of non-precipitated protein calculated. It can be seen from Figure 2.1 that by 20 minutes all but 17% had become particulate. Routinely, the IgG was incubated for 30 minutes at 60°C.

A second IgG1 myeloma (Mrs W) was prepared and heat treated in the same way. The solution became neither opalescent nor particulate although some aggregates were formed because it exhibited an anti-complementary activity of 1 in 32 (compared to 1 in 4096 for "Mrs H"). The conditions for aggregation were altered, increasing the protein concentration, the temperature and the length of incubation but no further changes were induced.

The degree of aggregation of gamma globulins is known to be variable and was studied in depth by Morse (1965). She isolated the gamma-globulin fractions from eighteen myeloma sera and two normal sera, as well as using a commercial source of pooled human Cohn fraction II. Each was heated for up to 15 minutes at 63°C and their anti-complementary activity and rheumatoid factor binding activity (by precipitation) measured. Representative samples were subjected to ultracentrifugation analysis to assess the amount of protein that had become aggregated.

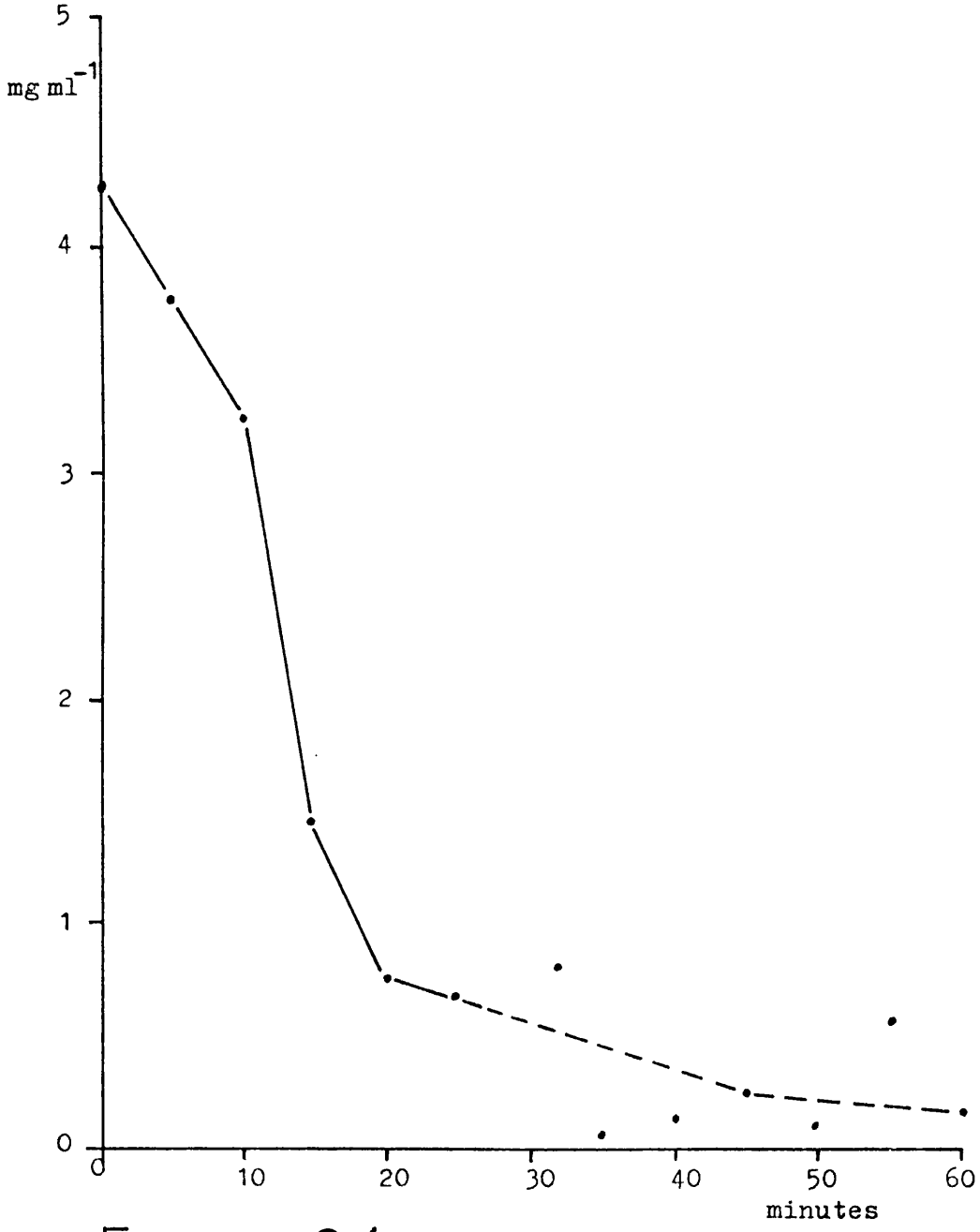

Figure 2.1

The aggregation of myeloma IgG.
ordinate: mg ml^{-1} of IgG remaining in solution.
abscissa: time of incubation, in minutes, at 60°C. Time zero equalled the point at which an equivalent solution reached 56°C.

I. Four of the eighteen myelomas became heavily opalescent at 1 mg ml^{-1} (and were complement-activating) and precipitated at 10 mg ml^{-1}. Ninety percent was aggregated after only 3 mins at 63°C.

II. Three of the eighteen became anti-complementary on heating at 10 mg ml^{-1}, at which concentration all were precipitated with rheumatoid factor. About 50% of one sample was seen to be aggregated upon ultracentrifugation analysis.

III. The larger proportion (11 of 18) became neither opalescent nor anticomplementary and contained no aggregates.

IV. The normal IgG's became opalescent and were 20% aggregated.

A full description has been given in order to indicate that "Mrs H" myeloma belonged to the first type while "Mrs W" IgG was apparently of the second, and contained some soluble aggregates.

The reason for the differing behaviour of the two preparations was not obvious. The aggutinability did not relate to either the overall charge (measured by starch block electrophoresis) or to four immunoglobulin allotypes (two Gm and two Km) (Morse, 1965). The knowledge that "Mrs H" myeloma was Glm (f) negative while "Mrs W" was Glm (f) positive (see Chapter 6) reinforces this finding.

6. Immune complex tests.

Anti-complementary activity (ACA) was measured by the Immunology Department, Royal United Hospital, Bath. Determination of % Clq-binding activity was carried out at the Blood Transfusion Service at Bristol, using the method outlined by Zubler and Lambert (1977).

7. Other serological tests.

Levels of C3 and C4, CH_{50} and IgM RF titre (Direct anti-globulin test) were kindly carried out by the Immunology Department, Royal United Hospital, Bath.

8. Statistical analysis.

Statistical significance was tested for by the Students t-test, using the small sample method for estimating the standard error after the variance ratio test had been performed. The t value was also calculated from a correlation coefficient. Chapters 13 and 16 of M.J. Moroney's "Facts from Figures" (1965) were used as a guide throughout this analysis.

CHAPTER THREE SEPARATION OF CELLS

Introduction

The isolation of leucocytes from human peripheral blood by density-gradient centrifugation techniques has largely superseded that of dextran sedimentation in which yield and purity were poor (Hjorth, Jonsson and Vretblad, 1981). The use of Isopaque-Ficoll to isolate the mononuclear cell fraction as perfected by Boyum (1974, 1977) has been extended to that of granulocytes (English and Andersen, 1974; Boyum, 1976) and monocytes (Gadeburg, Rhodes and Larsen, 1979), but the procedures involved may be lengthy(Pretlow and Luberoff, 1973; Loos and Roos, 1974; Loos *et al.*, 1976a).

Concurrent to Boyum's work was that of Pertoft, who developed a colloidal silica sol coated with polyvinylpyrrolidone as a density-gradient medium (Pertoft, Back and Lindahl-Kiessling, 1968; Pertoft *et al.*, 1977). This is now marketed under the trade name of 'Percoll' by Pharmacia Ltd, and has proved to be very versatile in allowing the isolation of particles ranging from viruses to macrophages (Pharmacia Ltd. literature), of lymphocytes and monocytes (Gutierrez *et al.*, 1979; Pertoft *et al.*, 1980; Gmelig-Meyling and Waldmann, 1980), granulocytes (Segal, Fortunato and Herd, 1980) and eosinophils (Gartner, 1980).

This medium was thus considered in order to harvest both monocytes and granulocytes from the same sample of blood: a bi-farious procedure was developed. This involved the use of a two-step gradient to effect the dissociation of granulocytes

from mononuclear cells, followed by sedimentation of the lymphocytes from the latter through a cushion of less dense Percoll.

Materials and Methods.

Choice of centrifuge tube;

All gradients and subsequent washes were performed in round-bottomed, screw-cap polycarbonate tubes (Nunc) to minimize cell adherence. This material was chosen because cultured cells have been shown not to adhere to polycarbonate suspension culture vessels (Cook, Counter and McColley, 1976), and unlike polypropylene does not have the disadvantage of being opaque.

Centrifugation of gradients was carried out in a temperature-controlled Mistral 6L centrifuge (MSE) at 18°C, and washes in a Heraeus-Christ 6000 bench centrifuge.

Preparation of gradients.

Percoll, purchased from Pharmacia (Great Britain) Ltd., Middx., was prepared as follows:
The stock solution of Percoll has a density of 1.130 ± 0.005 g ml^{-1}. Nine parts of this were mixed with 1 part of 10x PBS to give the working solution (WS), which was further diluted in 1xPBS to give the required densities, which were determined using a torpedo-shaped hydrometer graduated from 1.050 to 1.100 g ml^{-1} (Baird and Tatlock). These agreed closely with those calculated from the known densities of the buffers and stock Percoll according to the equations given by Pharmacia themselves (Percoll,

Methodology and Applications).

Two-step gradients were generated as follows:

2 mls of the denser of the two solutions (designated Y) were pipetted into a polycarbonate tube which was held upright in a clamp. A rubber collar or metal clamp was attached to the barrel of a 2 ml plastic syringe on which was a Gauge 21 needle, so that when inverted the syringe would rest inside the tube with the needle-point just above solution Y (Figure 3.1). 1.6 mls of the lighter solution X was drawn into the syringe which was then inverted, as the plunger was removed, into the tube. The upper layer of the gradient formed without disturbance of the lower, and could be left while other gradients were generated in the same way.

Use of gradients.

Citrated peripheral blood diluted 3 in 5 with iso-osmotic PBS could then be layered on top of solution X, using a Pasteur pipette that had previously been angled in a Bunsen flame (Figure 3.1). The filled tubes were spun at 400 g for 25 mins, and the cells at the two interfaces harvested by aspiration from above with an ordinary Pasteur pipette. Those from the upper layer were washed once in PBS containing 0.2% Egg Albumin (PBSA) (at 400 g for 10 mins.), and resuspended in 1 ml of this, to be layered onto a 2.5 ml cushion of a less dense Percoll. This was subjected to 750 g for 30 mins., and the interface cells collected. In some cases the pelleted fraction was also processed.

Legend to Figure 1.

The procedure for preparing a two step gradient of Percoll.

Illustration A: Polycarbonate tube held vertical in a clamp, and containing 2 mls of the denser solution Y.

B: A 2 ml plastic syringe with a small clamp around upper part, and containing 1.6 mls of the lighter solution X.

C: The very end of a pasteur pipette is allowed to bend over the gentle flame of a Bunsen burner.

D: The clamp around the syringe allows it to rest in the tube, the tip of the needle just above the surface of Y, and against the wall. Solution X flows in freely without disturbing Y.

E: By the same principle, the blood/ buffer mixture can be layered onto the gradient with the tip of the curved pipette against the wall of the tube.

facing 61

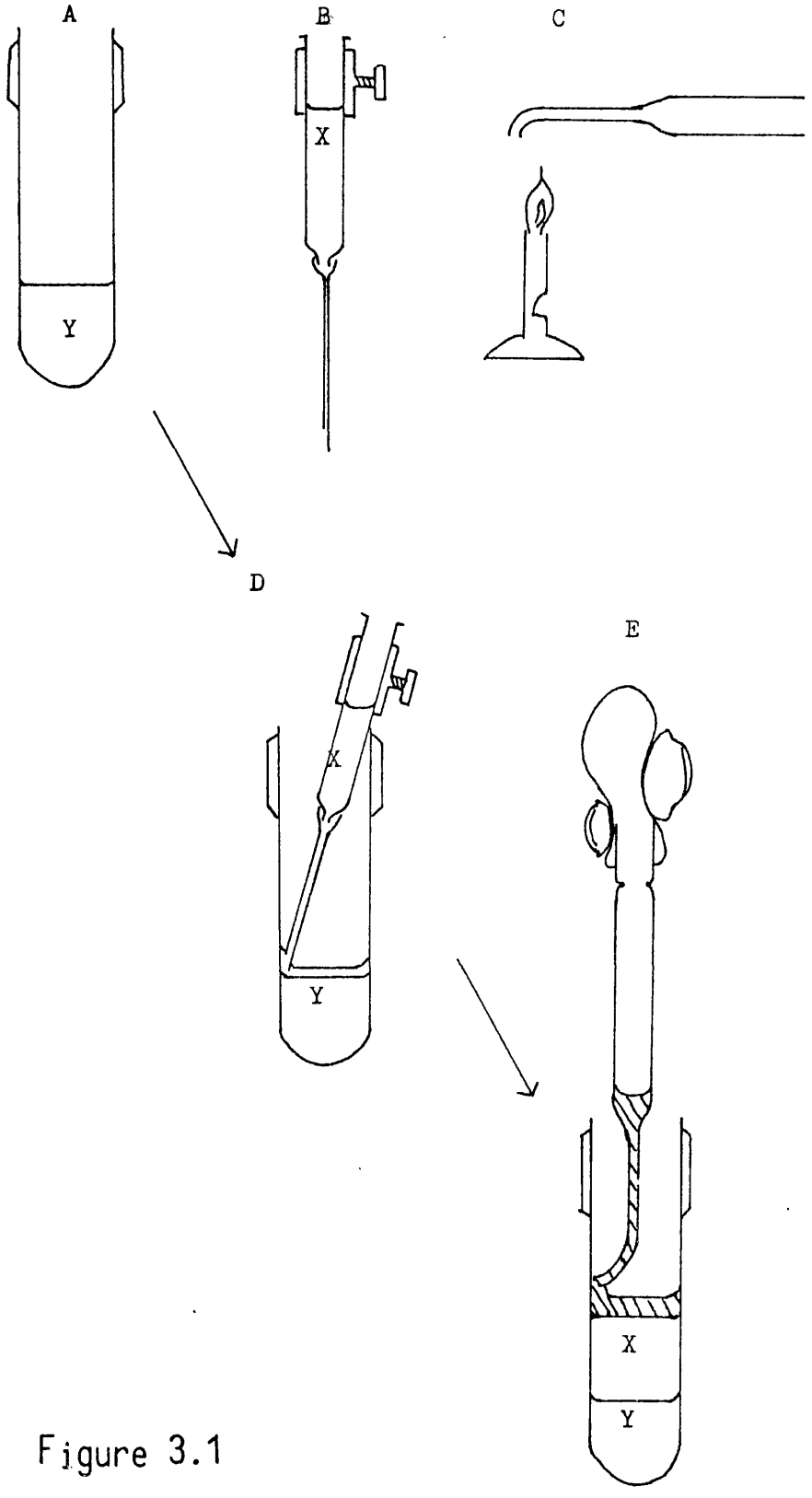

Figure 3.1

All fractions were washed thrice in PBSA before being re-suspended in the appropriate medium and counted. Cytocentrifuge smears were prepared for differential counts.

Use of an alternative method.

In some experiments the separation of granulocytes from mononuclear cells was carried out using Ficoll-Paque (Pharmacia) according to the method of Boyum (1974), and the contaminating erythrocytes lysed by exposure to buffered ammonium chloride, as detailed in Section 2.1.

Isolation from synovial fluid.

Granulocytes were also isolated from synovial fluid as follows. The synovial fluid was taken into preservative-free heparin (125 U per 10 mls.). Hyaluronidase (Sigma) was added to the fluid at 0.3 U ml^{-1} and allowed to react at 37°C for 30 mins. (Frøland, Natvig and Husby, 1973), after which the fluid was diluted with an equal volume of PBS and layered onto Ficoll-Paque (Pharmacia). For the sake of comparison, the peripheral blood was also heparinized, diluted volume for volume with PBS and separated on Ficoll-Paque as mentioned above. The pellet below the Ficoll was washed once and the erythrocytes lysed with buffered ammonium chloride. The synovial fluid granulocytes did not always require this treatment. The granulocytes were washed a further twice before being resuspended in M 199 plus 10% heat-inactivated foetal calf serum.

Trisodium citrate was employed as the anti-coagulant in

all but the above separation because the monocytes were found to clump less than when heparin was chosen. This finding has also recently been published by Brandslund *et al.* (1982) who noticed a marked aggregation of thrombocytes, with monocyte involvement, in the presence of heparin.

Results

For the first stage, that is the two-step gradient, the dilutions of the working solution of Percoll which proved to be the most useful were 65% for solution X (density 1.083) and 76% (ρ= 1.096) or 77% (ρ= 1.097) for solution Y. Further experimentation showed that the yield was better with the latter (Figure 3.2) (78% against 62%).

This gradient gave a mononuclear cell layer free of all granulocytes save for an occasional myelocyte, and a neutrophil layer containing a few eosinophils.

The separation and cell yield was similar for both normals and patients suffering from rheumatoid arthritis, Erythrocyte contamination of the granulocyte layer was variable and higher in the patient group but acceptable since treatment with buffered ammonium chloride for ten minutes between the first and second washes effected their removal.

The viability of all cell types, as measured by trypan blue dye exclusion, was greater than 99%.

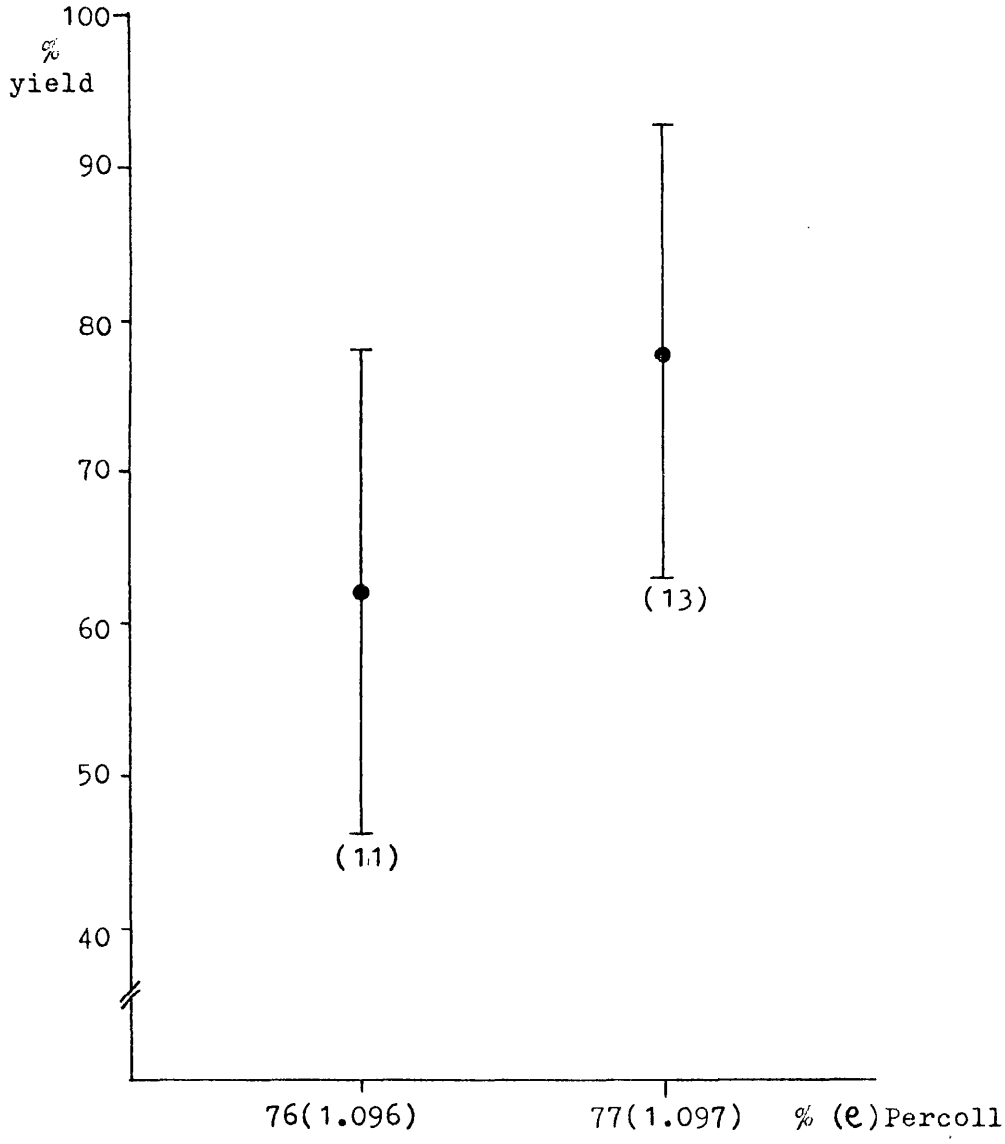

Figure 3.2

The yield of neutrophils gained by using two different Percoll densities.

Results expressed as mean ± one standard deviation, with the number of samples in brackets.

The difference between the two groups was not significant.

For the second stage of cell fractionation, a cushion of 52% Percoll (c. 1.068) was chosen as recommended by Carter (Pers. Commun.). The supported layer contained between 50 and 100 percent monocytes (as judged by their morphology) and the pellet 80 - 100 percent lymphocytes. After the subsequent washes, the viability of the monocytic cells was in the order of 95% and the average yield 78% of those present in peripheral blood. A differential count was always performed and the cells suspended to a constant number of monocytes per ml of medium.

A summary of the technique is given in Figure 3.3.

Discussion

The method described for isolating granulocytes has been used regularly for over two years and has consistently given good yields and pure preparations with high viability. The cells have been shown to be able to release free oxygen radicals and to be reactive in phagocytosis assays.

This method is very similar to that recently published by Hjorth, Jonsson and Vretblad (1981), although there is a difference between the densities of Percoll chosen for the separation. The density chosen by Hjorth *et al.* to isolate the mononuclear cells (equivalent to Solution X in Figure 3.1) was the same as that of Ficoll-Paque (1.0776 g ml^{-1}) whereas the protocol outlined in this chapter required a density of 1.083. The medium for the granulocyte-erythrocyte separation was likewise of

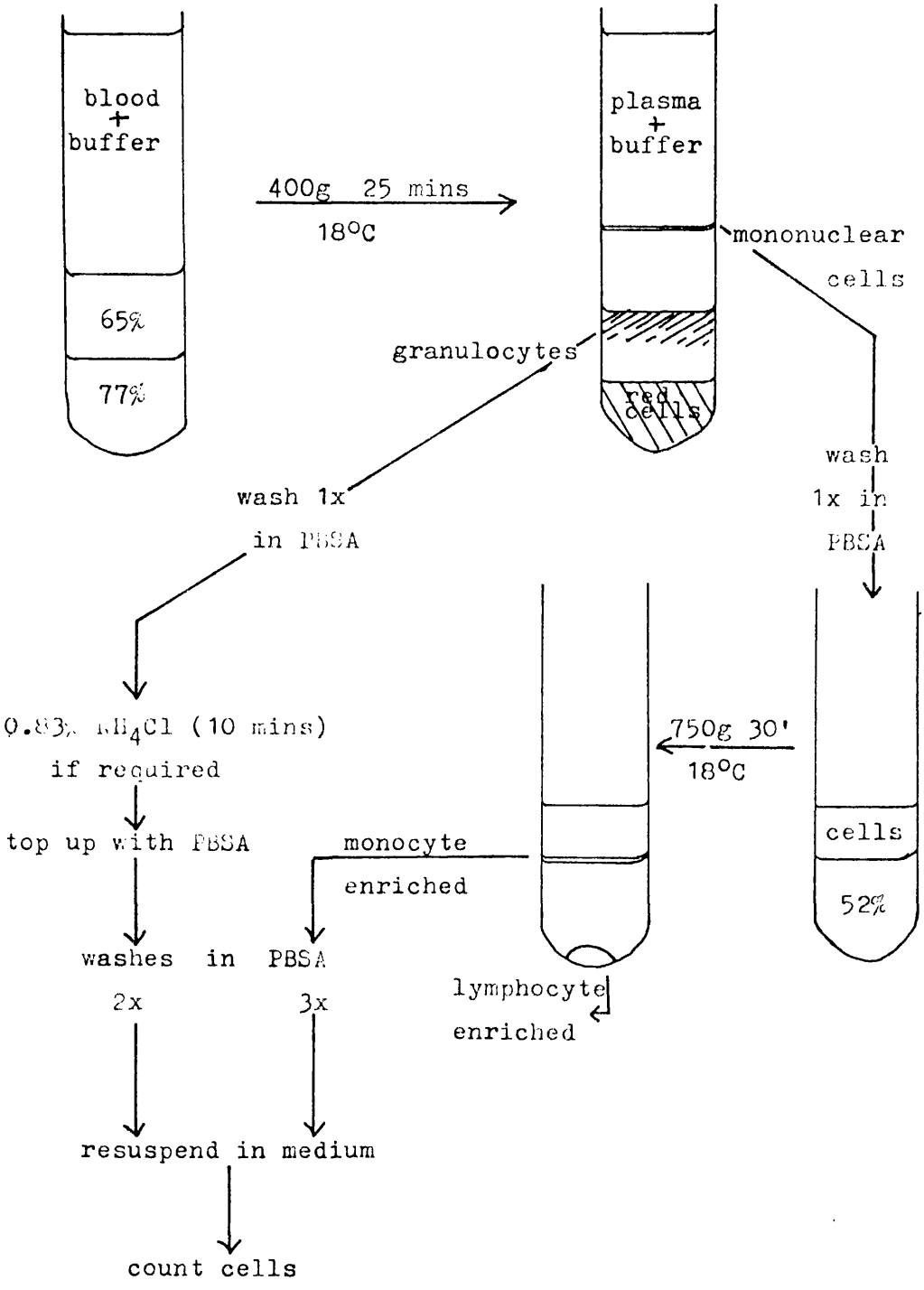

Figure 3.3

Scheme for isolating cells.

different density, in this case less dense than Hjorth's; 1.097 compared to 1.1026. Tube size and g forces were similar. The reason for these differences is unclear. Some experimenters , including those mentioned above, advise the layering on of whole blood (Pertoft *et al.*, 1980; Hjorth, Jonsson and Vretblad, 1981) while others, including Boyum (1976) advocate dilution with buffer, a procedure followed here. Fractionation behaviour has been reported to vary with the haematocrit of the blood applied (Pertoft *et al.*, 1980); this dilution step may therefore be an important factor in rationalizing the different conditions.

Another procedural difference was that while the stock Percoll was made isotonic by dilution with ten-times PBS as suggested by Pharmacia (Percoll, methods and applications), Hjorth and colleagues added solid sodium chloride to the Percoll. Even slight changes in tonicity can drastically alter cell separation (own observations, unpublished) and should therefore be considered of importance.

Gartner (1980) has combined dextran sedimentation with a five-step gradient to isolate eosinophils in addition to neutrophils and mononuclear cells. The need for an ultracentrifugation step has also been circumvented by Segal, Fortunato and Herd (1980) whose method utilizes a perspex gradient mixer to combine the blood and Percoll. All have found a high viability and integrity of cellular function after isolation using Percoll. Pertoft and his colleagues (1977) incubated

cultured cells in various density gradient media and found that Percoll has little effect on the growth characteristics even after 100 hours, and much less so than did Ficoll.

The decreased separation of erythrocytes from granulocytes in rheumatoid patients' blood, noticed in this study, has also been recently reported by Ferrante *et al.* (1982). This group of workers found it advantageous to alter their separation media accordingly. This approach was not considered necessary since very few samples did not separate well enough to be processed.

The densities of monocytes and lymphocytes overlap considerably, so that the recovery of the two types on Percoll (or Ficoll) can suffer from cross-contamination, a phenomenon which seems to be donor-dependent (Gmelig-Meyling and Waldmann, 1980; Pertoft *et al.*, 1980). The purity and yield gained with the 52% Percoll was comparable with that found by Gmelig-Meyling and Waldmann (1980) and by Brandslund *et al.* (1982); lower yields but higher purity were experienced by Pertoft (1980). The density of the separation medium was similar in all cases. The combination of buoyant density centrifugation with adherence to glass or plastic may give a yield as low as 9% (Gadeburg, Rhodes and Larsen, 1979) without a concomitant increase in purity (24%). However, pre-coating of plastic petri-dishes with foetal calf serum prior to allowing adherence of mononuclear cells, as developed by Kumagai and co-workers (1979), followed by detachment with a chelating agent, is capable of

giving much higher purity (96%) and yield (54%).

The use of such additional methods makes comparison of the monocytes with the neutrophils from the same blood sample more difficult. Primarily, the additional technique may alter the functional capacity, or the surface, of the cell; secondly, the use of multiple techniques within the purification procedure may lead to decreased cell responsiveness because of the increased time factor before use. This is especially true for neutrophils (Ferrante, Beard and Thong, 1980) which have to be kept until the monocytes have been purified.

The mononuclear cells can be contaminated by myelocytes and myeloblasts, as noted by Boyum (1976). In normal controls, this is unimportant; they were occasionally found in preparations from rheumatoids, but never more than one percent.

This method was designed to subdivide peripheral blood leucocytes so that both a monocyte-enriched and a granulocyte population could be studied in parallel. It has proved to be functional and effective.

CHAPTER FOUR. PHAGOCYTOSIS I: THE CHOICE OF STIMULUS AND ASSAY SYSTEM WITH A VIEW TO MEASURING PHAGOCYTOSIS.

Introduction

It was decided to study the properties of the peripheral blood phagocytes because of their importance in inflammation. It is known that exocytosis occurs during the phagocytic event if the stimulus is large or surface bound because this cannot be fully phagocytosed (Henson, 1977; Treadway *et al.*, 1979). This phenomenon is a useful tool in the measurement of phagocytosis. The effects of both soluble and insoluble heat-aggregated IgG have been studied extensively (Henson, Johnson and Spiegelberg, 1972; Henson and Oades, 1975; Treadway *et al.*, 1979), as have precipitated and soluble immune complexes (Ward and Zvaifler, 1973a and 1973b; Turner *et al.*, 1976; Lamers, 1980); these aspects are elaborated upon in the discussion of this chapter and in Chapter 6. Three particulate and two soluble compounds were chosen as possible agents to cause degranulation and their suitability compared.

Phagocytic cells contain a wide range of lysosomal enzymes (Bretz and Baggiolini, 1974; Nathan,Murray and Cohn, 1980; Segal, Dorling and Coade, 1980) which can be assayed spectrophotometrically. The acid hydrolases can break down a p-nitrophenyl or phenol-phthalein labelled substrate; myeloperoxidase, which may be located in a different azurophil granule to the acid hydrolases (Segal, Dorling and Coade, 1980) can be measured by its ability to oxidize O-dianisidine. The cytoplasmic enzyme LDH is released on cell death, and is assayed by its mediation of the oxidation of NADH to NAD as pyruvate is converted to lactate.

Materials and Methods

Heat aggregated human IgG1 was prepared as described in Section 2.3 as was soluble aggregate. Latex beads LB11, diameter 1.091 ± 0.0082 μm (Sigma) and carbonyl iron powder, Type SF (GAF (GB) Ltd., Manchester) were not treated or washed prior to use. Soluble complexes were partially purified from rheumatoid synovial fluid (see Section 2.5 (iv)) and showed an ACA of 1/512 in the system used, at a protein concentration of 2.9 mg ml^{-1}. A plasma containing soluble complexes were also studied, from a patient (initials D.C.) with RA and associated diffuse vasculitis who had an ACA of ½, IgM RF of 1/512 and a Clq binding of 94.9%. Serum was generated from this by the addition of a three-fold excess of calcium and magnesium chloride (calculated with respect to normal plasma levels). The clot was allowed to retract overnight at 4°C before the serum was harvested.

The granulocyte and mononuclear phagocytes were prepared by the method given in the previous chapter (Chapter 3) and were from apparently healthy donors unless otherwise stated. Monocyte enriched populations were not prepared during this part of the study. The phagocytic conditions have been set out in the 'Methods' fascicle (Section 2.3) and details of the enzyme assays are also given there in Table 2.1. The time course experiments were stopped by the addition of an equal volume of cold medium, so that the volume that was added to the enzyme assays was twice that of the usual. The amount of enzyme released was expressed as a percentage of the

total in an unstimulated cell (LDH) or of that remaining in the cell plus that released (acid hydrolases).

That is, if R = amount released

and Rm= remainder (cell lysate)

Then $$\% \text{ released} = \frac{R \times 100}{Rm + R}$$

Myeloperoxidase was expressed in terms of the equivalent activity of the standard horseradish peroxidase (Type II, 165 PGU, per mg solid. Sigma). Standards were employed for the acid hydrolases and the LDH in order to check the behaviour of the assays that were used.

Results

Choice of stimulus

The release of three different acid hydrolases was measured in the absence of any stimulus and in the presence of 500 μg ml^{-1} of the aggregated IgG for both a granulocyte (PMN) preparation at 5×10^6 cells ml^{-1} and a mononuclear cell (MNC) adjusted to 5×10^6 monocytes per ml. After one hour's incubation at 37°C, the background release of β-galactosidase ranged from zero to 65% for MNC (n = 5) and to 67% for PMN (n = 5), with the increment induced by stimulation varying between seven and forty-four percent. The exocytosis of both acid phosphatase and β-glucuronidase was much less variable, and is shown in Figure 4.1. Each population of cells did not release equivalent levels of both enzymes. Lymphocytes alone did not release these enzymes (results not shown). A small number of experiments was carried out to assess the response of granulocytes to carbonyl iron and to latex. The former was used

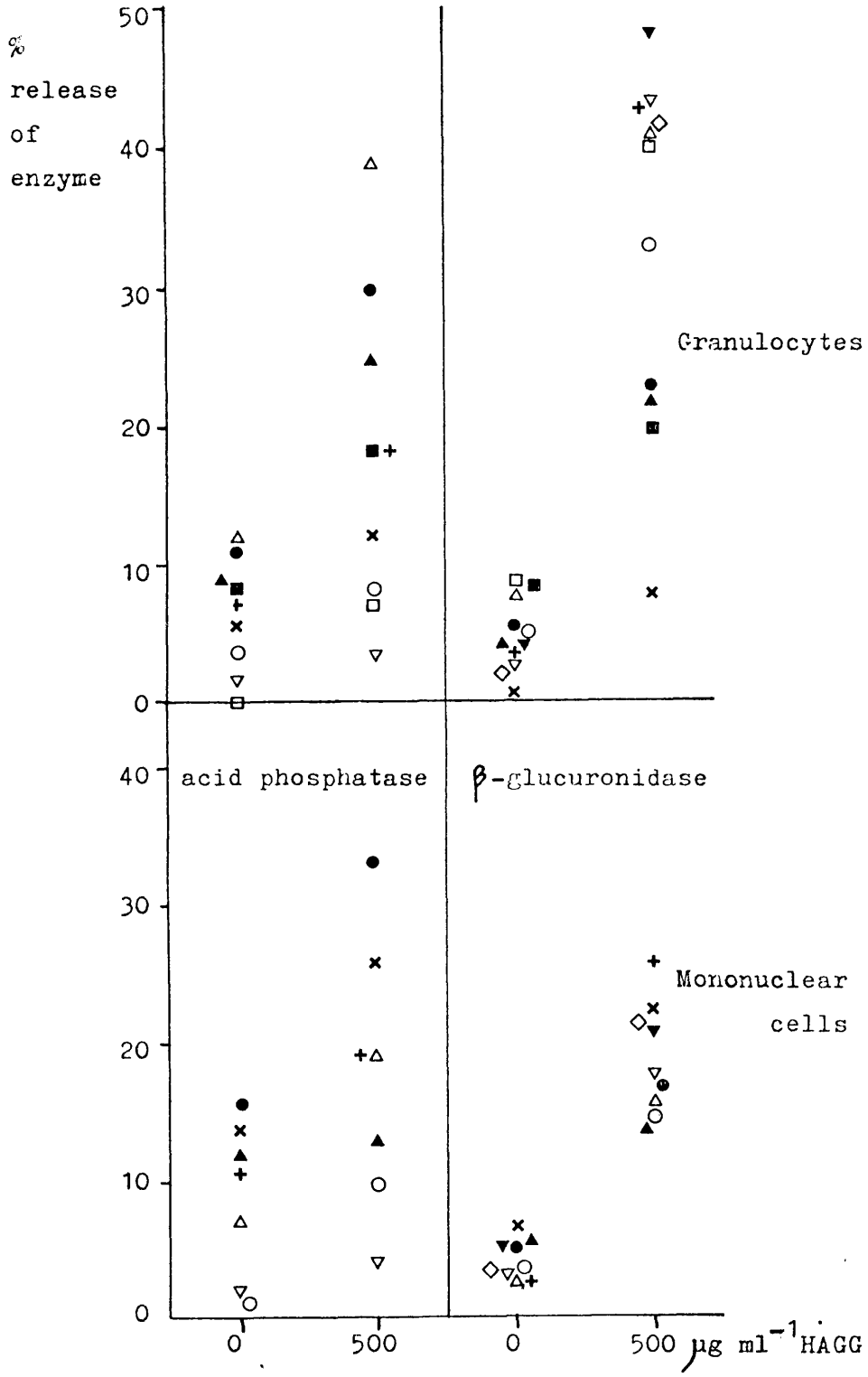

Figure 4.1

The response of phagocytes towards aggregated IgG (HAGG), measured by the release of lysosomal enzymes. Granulocytes were at 5 x 10^6 ml^{-1} and monocytes at the same except for × and + which were at 2 x 10^6 ml^{-1}. Incubation time was one hour at 37°C. Each value is the mean of duplicate samples.

at 200 μg ml^{-1}, and the latter at a latex:cell ratio of 10:1. This is detailed inFigure 4.2 which shows that neither particle promotes any enzyme release. One experiment was carried out with MNC and it also reflected this pattern.

The immune complex-containing IgG fraction from synovial fluid was compared with the myeloma IgG that had been passed through a Sepharose 6B-Cl column to remove aggregates, and also with HAGG. A definite release of lysosomal constituents was noted in response to exposure to the synovial fluid IgG and this is shown in Figure 4.3. Further, a comparison was made between this SF IgG, the HAGG,and soluble aggregate with both granulocytes and mononuclear cells, and the results are presented in Figure 4.4. Both mononuclear cells and granulocytes responded to the SF IgG, but to a lower degree than to HAGG ($.05 < P < .1$ and $P < .001$ respectively). Monocytes failed to degranulate in response to soluble aggregates ($P < .001$ cf. HAGG) while granulocytes responded poorly ($P < .001$ cf. HAGG). The difference in response to soluble aggregate and synovial fluid IgG was significant in monocytic cells ($0.01 > P > .001$) and approached significance with PMN ($.1 > P > .05$).

The use of a whole plasma that contained immune complexes, for a phagocytic assay, created two major problems . The plasma level of β-glucuronidase was much higher than the expected release from the cells. Once this background had been subtracted, any changes that might have occurred appeared negligible. If the plasma was diluted in tissue culture medium in order to diminish this problem, the divalent cations in the medium induced the plasma to

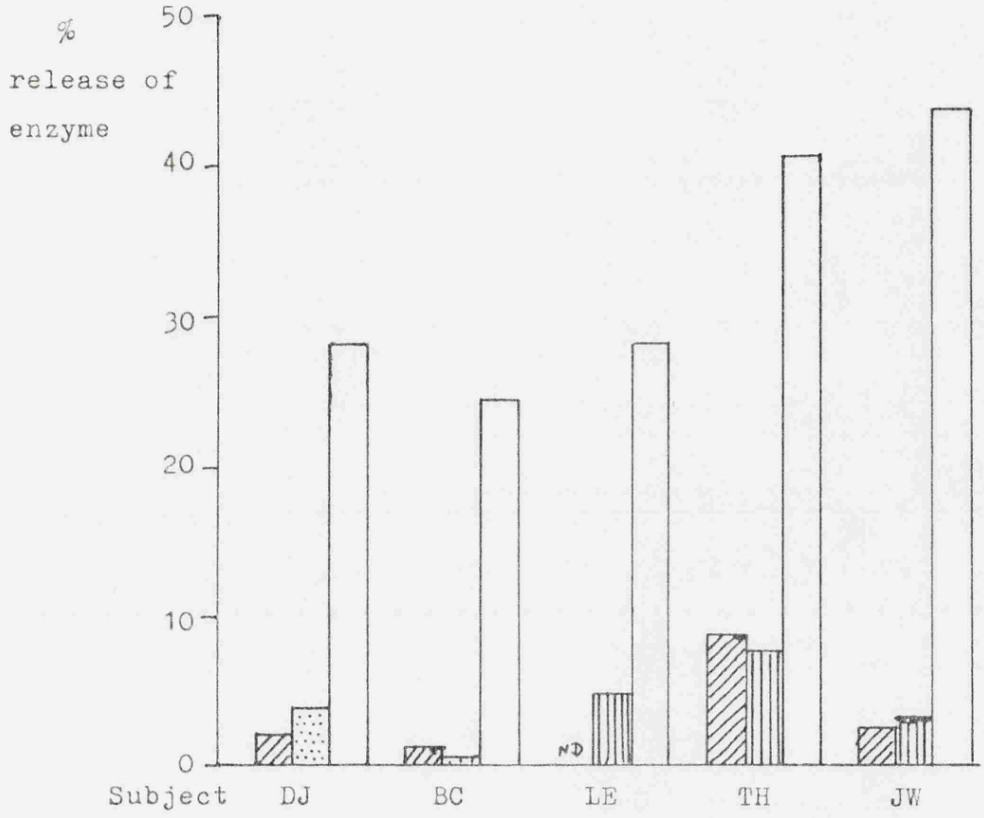

Figure 4.2

The release of β-glucuronidase in response to stimuli, using granulocytes.

Open bars: the response of each subject's cells to 500 $\mu g\ ml^{-1}$ HAGG.

Hatched bars: release in the absence of stimulus.

Stippled bars: release with latex at 10:1 (latex:cell), DJ and BC only.

Vertical bars: release with carbonyl iron at 200 $\mu g\ ml^{-1}$, LE, TH and JW only.

Each test was performed in duplicate.

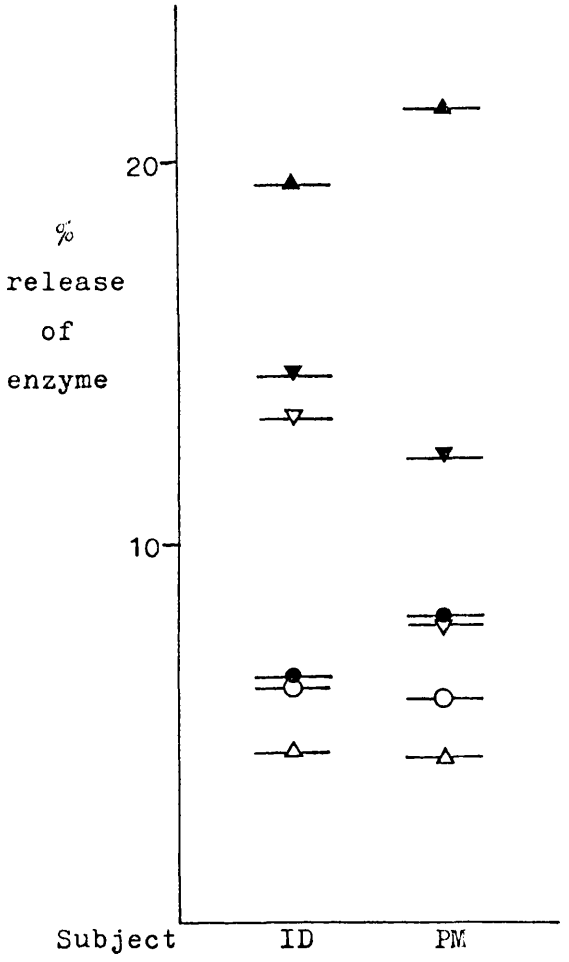

Figure 4.3

The release of β-glucuronidase induced by synovial fluid IgG containing complexes, in comparison to the activity of monomeric and aggregated IgG.

No stimulus △

IgG, 1.25 mg ml^{-1} ○

IgG, 1.5 mg ml^{-1} ●

S.Fluid IgG, 1.25 mg ml^{-1} ▽

S.Fluid IgG, 1.5 mg ml^{-1} ▼

Aggregated IgG, 1 mg ml^{-1} ▲

Each experiment was performed in duplicate on granulocytes.

Figure 4.4.

The release of β-glucuronidase (background subtracted) in response to:

500 μg ml^{-1} soluble aggregate (Sol Agg)

500 μg ml^{-1} synovial fluid IgG (SF IgG)

200 μg ml^{-1} heat-aggregated IgG (HAGG)

by mononuclear cells (1.25 x 10^6 ml^{-1} monocytes) and granulocytes (5 x 10^6 ml^{-1}).

Statistical analysis;

All results are presented as mean ± 1 standard deviation.

n = 6 for both HAGG and Sol Agg groups

n = 5 for SF IgG group.

Pair	MNC	PMN
HAGG: Sol Agg	$P < 0.001$	$P < 0.001$
HAGG: SF IgG	$0.1 > P > 0.05$	$P < 0.001$
Sol Agg:SF IgG	$0.01 > P > 0.001$	$0.1 > P > 0.05$

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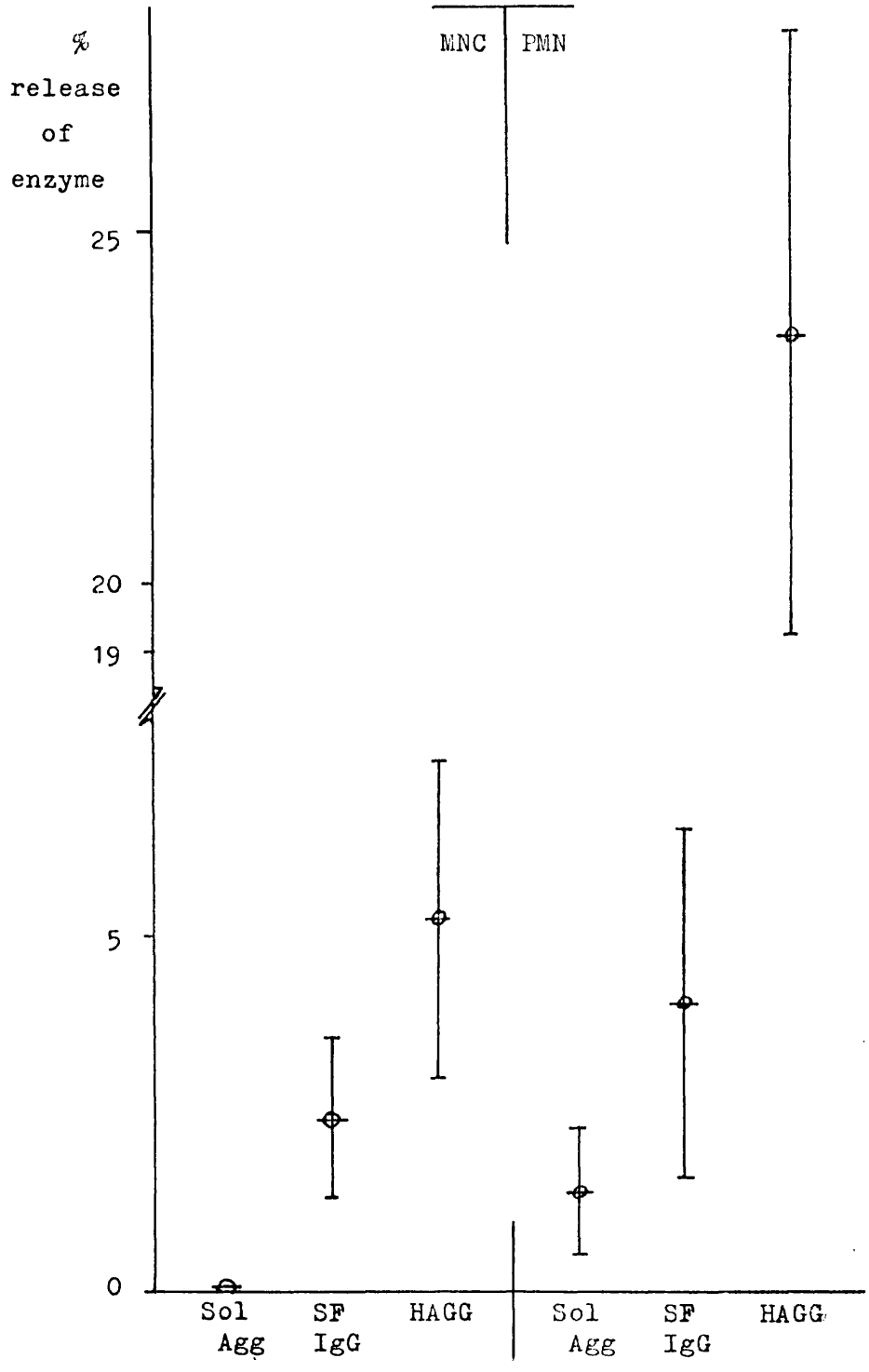

Figure 4.4

The release of lysosomal enzyme in response to various stimuli.

See legend opposite.

clot. Some of the plasma was, therefore, clotted before use as detailed in the Methods to this chapter. This serum was diluted from 100% through to 10% but none of the dilutions induced a measurable release of β-glucuronidase by granulocytes or monocytes (two subjects). The same result was obtained when the cells were incubated in neat plasma (5 subjects). This treatment did not cause any cell death as assessed by trypan blue exclusion before and after the incubation period.

Lysosomal enzymes.

Since acid phosphatase and β-glucuronidase are both acid hydrolases, it was decided to also follow the exocytosis of peroxidase. This release was of the order of 10^{-3} purpurogallin units (PGU) per 2.5 x 10^{6} cells per ml. The initial experiments suggested that the kinetics of release in this system were subject-dependent, but appeared to plateau by 20 mins for peroxidase while possibly still rising for β-glucuronidase release (Figure 4.5). In further experiments with longer incubation times these possible differences were not consistently shown: Two patients with RA and 2 normal controls were studied for both cell types (in duplicate) and for both of the enzymes, and representative data from granulocyte populations are shown in Figure 4.6. Subject N.F. showed a plateau for both enzymes, whereas the release of β-glucuronidase continued to rise with C.B. cells. The mononuclear cell preparations failed to respond with peroxidase release but did so with β-glucuronidase. The time course for this release was similar to that found for the granulocytes shown in Figure 4.5., except that the release tended to plateau between 5 and 15 minutes before rising further (results not shown).

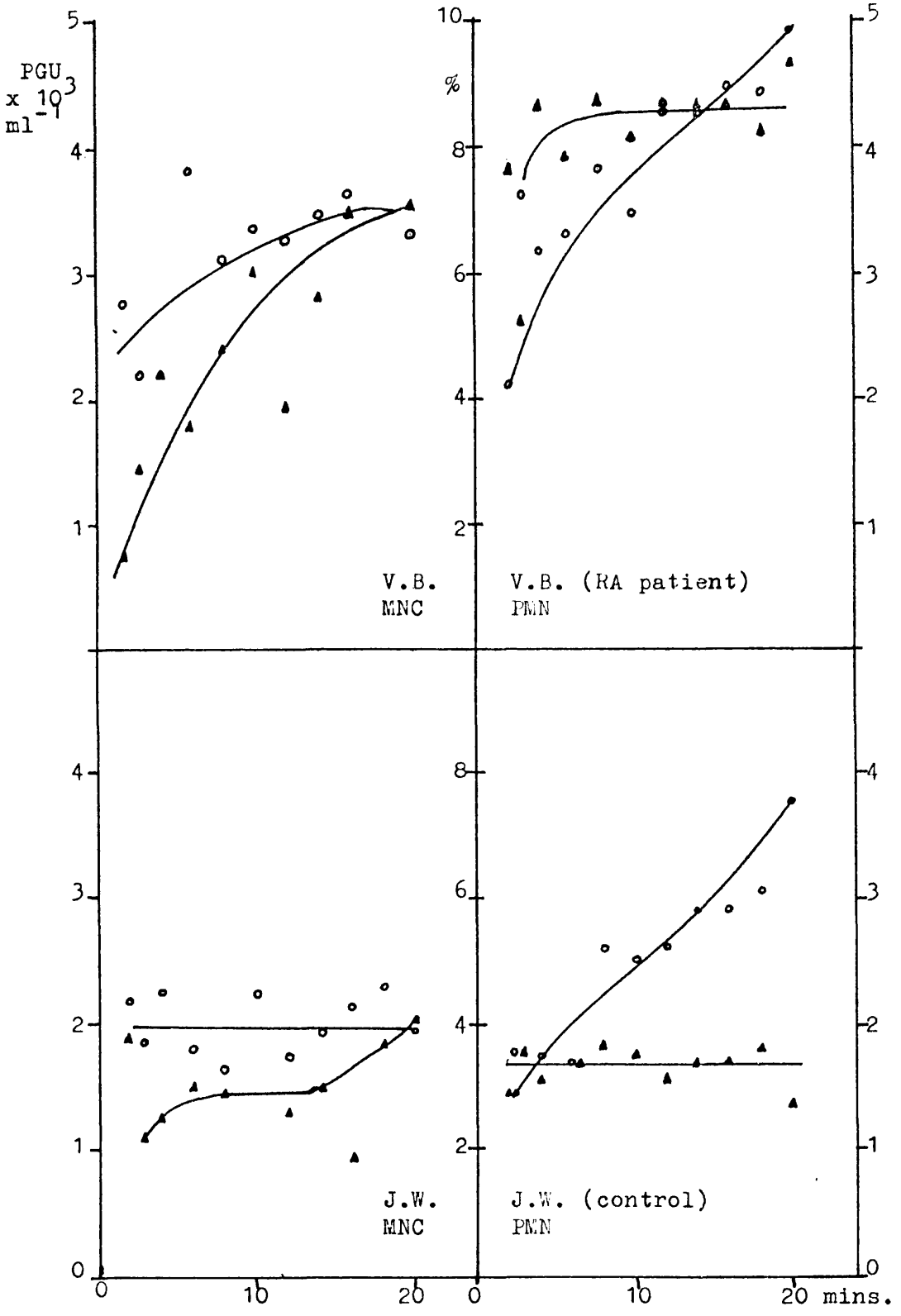

Figure 4.5

A comparison of the release of two lysosomal enzymes in response to 300 μg ml^{-1} HAGG, over time.

Release of peroxidase ▲ - lateral ordinates, PGU x 10^3/ml

Release of β-glucuronidase o - middle ordinate., %.

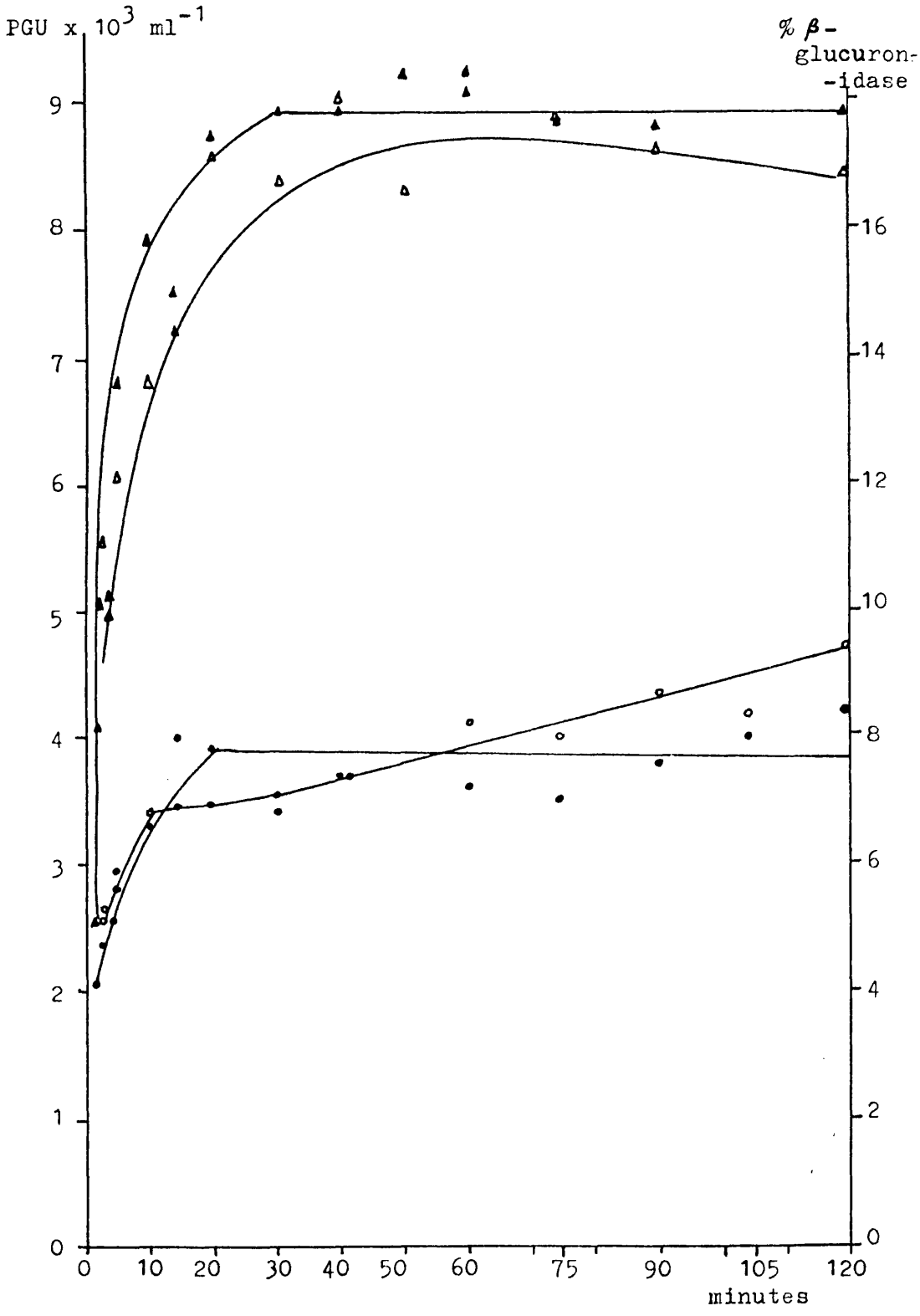

Figure 4.6

The response over 120 minutes of granulocytes exposed to 300 μg ml^{-1} HAGG.

Release of peroxidase ▲ β-glucuronidase Δ N.F.(control)

● ○ C.B.(patient)

Discussion

Insoluble aggregated immunoglobulin G has been shown to stimulate the release of β-glucuronidase from neutrophils (Henson, Johnson and Spiegelberg, 1972), and this has been demonstrated here to be true for mononuclear cells as well as for granulocytes. Evidence suggests that soluble aggregates or complexes do not induce exocytosis (Treadway *et al.*, 1979; Lamers, 1980) but do stimulate the phagocyte's hexose monophosphate shunt (HMPS) activity (Henson and Oades, 1975). It was therefore not surprising that the complex-containing plasma did not induce a measurable degranulation.

An unexpected finding was that the "immune complex" rich IgG from synovial fluid should have caused a degranulation, albeit less marked than with HAGG (see Figure 4.4.). The preparation had been stored at -90°C for two weeks prior to use, but the plasma mentioned above had also been kept at this temperature. A further gel-filtration step followed by the immediate use of various fractions and determination of their ACA might have helped to elucidate the problem. It has proved difficult to correlate biological activity and size. McCarthy, for instance, has reported that aggregates of the same size, carefully isolated by polyethylene glycol precipitation and subsequent sucrose gradient, behaved differently in each of the three assays used for their detection (McCarthy *et al.*, 1981).

Taichman, Pruzanski and Ranadive (1972) noted that a low, but variable, amount of degranulation could occur in the response of rabbit PMN to soluble albumin complexes, and this was found to be

true for human PMN using soluble aggregates of IgG (Figure 4.4). Monocytes, however, did not release enzymes under these circumstances, which may reflect the general lower responsiveness of monocytes, even to HAGG, under the experimental conditions.

Uncoated latex beads can be taken up by polymorphs (Hallgren, Hakansson and Venge, 1978) but did not cause any degranulation under the conditions used here. Carbonyl iron powder has been used to deplete MNC of monocytes because the latter ingest it and so become more dense. They can then be separated from the lymphocytes by density gradient centrifugation. No release of granular enzymes into the medium was noted when either granulocytes or MNC were exposed to carbonyl iron.

The acid hydrolases β-glucuronidase and acid phosphatase were both released in response to aggregated IgG, but a high release of one was not paralleled by the other. This suggests that the proportions of the two enzymes must be very different in either the granules of an individual cell or of the population as a whole. The higher background release of acid phosphatase, especially with MNC, meant that it was less suitable for evaluating the response of the cells to a stimulus. Myeloperoxidase may be contained along with the acid hydrolases in azurophil granules, or may be situated in a different type of granule (see Chapter 1, PMN leucocyte section). The release of peroxidase and β-glucuronidase seemed to parallel each other in these experiments. Measurement of a whole cell population is probably too insensitive a method to detect the difference in kinetics following phagosome fusion that

Segal, Dorling and Coade (1980) have noted using isolated vacuolar fractions. Both monocytes and granulocytes are peroxidase positive when this is demonstrated histochemically (Yam, 1971); the unresponsiveness of some of the mononuclear cell preparations was therefore unlikely to be caused by a lack of the enzyme.

From the results gained it seemed reasonable to follow the release of β-glucuronidase as a standard measure of degranulation in response to aggregated IgG.

CHAPTER FIVE PHAGOCYTOSIS II. COMPARISON OF THE LEUCOCYTES OF RHEUMATOID PATIENTS AND NORMAL CONTROLS

Introduction

Granulocytes and mononuclear phagocytes are important in inflammation, and for this reason the leucocytes from patients with rheumatoid arthritis (RA) have been compared with those from normal controls with respect to a number of parameters.

A defect in chemotaxis has been reported (Mowat and Baum, 1971) which seems to be attributable to the rheumatoid plasma (Walker, James and Smith, 1979), and more specifically to immune complexes Kemp *et al.*, 1979; 1980). Howe and fellow-workers could not, however, confirm these results; only the RA subgroup of Felty's patients exhibited a decreased neutrophil migration (Howe *et al.*, 1981). This was a cellular rather than a humoral phenomenon. The migration of a whole leucocyte preparation could be inhibited by rabbit/human IgG complexes if the cells came from rheumatoids but not from normals (Hall, 1978). Howe also noted that there were no significant differences between normals, rheumatoids and Felty's patients with respect to the cells' adhesiveness to a nylon fibre column (Howe *et al.*, 1981). Leucocytes from RA patients do seem to bind extracts of rheumatoid synovium as assessed by leucocyte adherence inhibition (Tannenbaum, 1979). Control cells were not affected.

The ability of monocytes to phagocytose bacteria (Bar-Eli *et al.*, 1980) and antibody-coated red cells (EA) (Temple and Loewi, 1977) was similar between patients and normals, although bactericidal

activity was diminished in cells from patients (Bar-Eli *et al.*, 1980). A defect in monocytic phagocytosis of serum-coated (that is complement-coated), killed yeast, has been demonstrated in patients suffering from cutaneous vasculitis in addition to rheumatoid arthritis, but not in those with RA alone (Hurst and Nuki, 1981). Both fresh normal serum and complement increased the binding of EA whereas seven sera from patients with systemic lupus erythematosus (SLE) exhibited inhibition. This was probably related to the presence of immune complexes (Temple and Loewi, 1977).

The uptake of IgG-coated latex by PMN has been reported to show a slight, but not significant, decrease for those from RA patients compared to normals (Hallgren, Hakansson and Venge, 1978). Yeast phagocytosis has been shown to be comparable (Turner, Schumacher and Myers, 1973) when using peripheral blood neutrophils but decreased in those isolated from synovial fluid, whether from rheumatoid or miscellaneous arthritides. Synovial fluids were less able to opsonize yeast for uptake than were sera.

More recently the secretory properties of peripheral blood granulocytes in response to zymosan were discovered to be the same in normals and in inactive patients who were receiving gold (van de Stadt, van de Voorde-Vissers and Feltkamp-Vroom, 1980). Blood and fluid granulocytes from patients with active synovitis (about half of whom were receiving second-line 'anti-rheumatic drugs') responded similarly to each other though synovial cells released more LDH than those from blood. The group with active synovitis was not directly compared to the normal controls.

It was decided to study the secretory capacity of both monocytes and granulocytes from patients with RA and from healthy controls. Aggregated IgG1 was used as the stimulus and β-glucuronidase as the indicator. Synovial fluid granulocytes were compared to those from paired peripheral blood samples in a separate study group.

Patients and Methods

The patients chosen had definite RA and at the time of bleeding had active synovitis. Eighteen out of 22 (82%) were seropositive by Rose-Waaler test and 10 of 22 (45%) showed some systemic disease such as nodules. One of these had definite Felty's syndrome, another possibly manifested this. The chemotherapy was confined to a wide range of non-steroidal anti-inflammatory drugs (NSAID) save for the possible Felty's patient who was taking 5 mg Prednisolone per day and a patient with pericarditis who was being treated with azathioprine. The duration of illness ranged from one to forty years, mean 12.4 ± 9.3 (± 1 Standard Deviation) and the age of the patients was from 33 to 75, mean 57.8 ± 11.3 years. There were 14 females and 8 males.

A small group of patients used as "ill controls" were being treated with NSAID but did not have RA. One (61 yrs, 4 yr illness, F) had severe osteo-arthrosis and four (1F, 3M) had ankylosing spondylitis (age 34 to 42, duration 13 - 20 years).

Synovial fluid studies were carried out on ten patients with RA with active synovitis. There were 6F and 4 M, ages 33 to 79; four were being treated with '2nd line' drugs in addition to NSAID (see

Table 5.1).

The control group were apparently healthy members of staff of the hospital. Their ages ranged from 22 to 61, mean 39.4 ± 14.0 and there were 12 females and 10 males. The controls for the drug experiment were made up of 4 F and 5 M, age range 25 - 42, and all were medical and scientific staff.

Methods.

The granulocytes (PMN) and monocyte-enriched cells (MEP) were prepared from citrated peripheral blood and synovial fluid granulocytes from heparinized fluid as described in Chapter 3. Three patients' fluids and bloods were separated on Percoll (patients E.H., L.B., and G.M., Table 5.1). The peripheral blood granulocytes of one patient (E.C.) were treated with hyaluronidase after their second wash, at a concentration of 0.3 U ml^{-1} of the original blood volume, and then washed once. White cell and differential counts were always carried out to check for abnormalities.

The phagocytic assays were carried out as described previously; with granulocytes at a concentration of 5×10^6 ml^{-1} and the monocyte-enriched cells adjusted to 1.25×10^6 monocytes ml^{-1}. The β-glucuronidase assessment was carried out as in Chapter 2 (Table 2.1). Each test was performed in duplicate and the mean taken.

Results.

The PMN and MEP preparations were exposed to concentrations of the IgG aggregate which varied from 50 to 500 μg ml^{-1} and compared

with the release at zero concentration. This background level was subtracted from each result which was then expressed as Δ% release of β-glucuronidase; this was also done on all subsequent data unless otherwise stated. Some subjects' cells were not challenged with every concentration of stimulus. Figures 5.1 and 5.2 show the results obtained for granulocytes and monocytes respectively. It can be seen that there were significant differences found between normals' and patients' granulocytes, the patient response being less than that of the controls. The most significance $p < .001$ was found in the cells' response to 300 μg ml^{-1} HAGG, controls releasing 24.6 ± 5.1% and patients 17.1 ± 3.6% glucuronidase (mean ± 1 S.D.). At all other aggregate concentrations tested, the differences had P values between 0.01 and 0.001.

The monocytes, however, behaved comparably over this time scale with no significant differences. It was found, as might be expected from the latter, that the degree of response of the two cell types did not correlate (for controls, $r = 0.163$, $n = 22$; for patients $r = 0.268$; $n = 21$).

Investigation of the difference.

Because of the differences in ages of the two groups as a whole, regression analysis was performed for the two groups. Age was compared with Δ% release at 200 μg ml^{-1} HAGG, There was a slightly decreased release with age but this did not approach significance ($r = -0.15$ for both controls ($n=32$) and patients ($n=32$).

Legend to Figures 5.1 and 2.

The release of β-glucuronidase in response to HAGG.

Ordinate: Release of enzyme with background (zero HAGG) value subtracted

Abscissa: increasing concentrations of HAGG.

Results expressed as $\bar{x} \pm 1$ SD

control group -O-

patient group -Δ-

Figure 1 appertains to granulocytes, $12 \leq n \leq 22$

Figure 2 appertains to monocytes (MNC) $10 \leq n \leq 22$

Statistical analysis, difference between patient and control groups;

HAGG	PMN	MNC
50 μg ml^{-1}	.01 > P > .001	not. sig.
100	.01 > P > .001	NS
200	.01 > P > .001	NS
300	P < .001	NS
400	.01 > P > .001	NS
500	.01 > P > .001	NS

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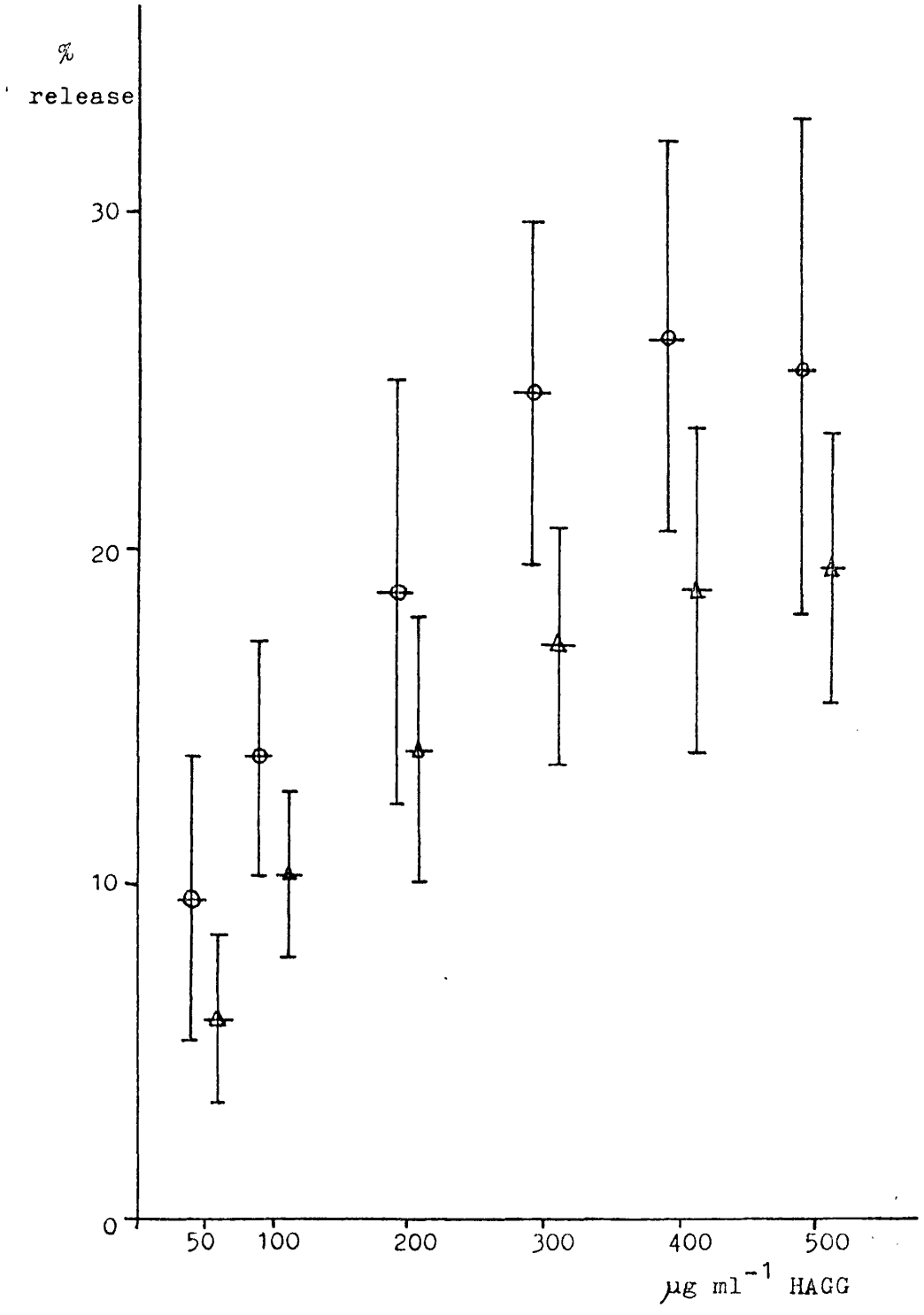

Figure 5.1

The release of β-glucuronidase by granulocytes.

See legend opposite.

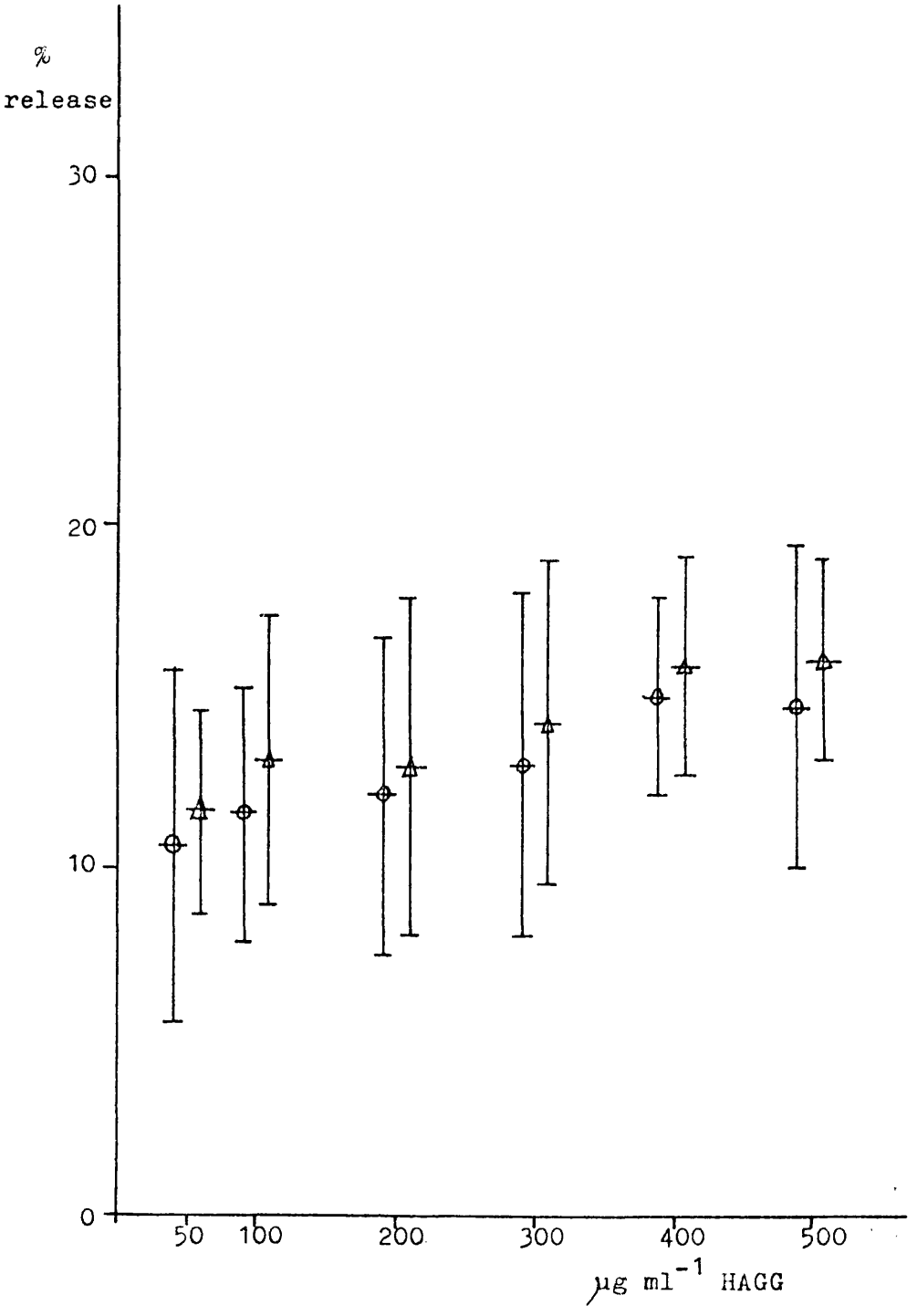

Figure 5.2
The release of β-glucuronidase by mononuclear cells.

See legend opposite Figure 1.

There was the possibility that the drug therapy was causing the depression of response, and that in this case the monocytes were less sensitive to its effects than were the neutrophils. Therefore, the behaviour of the granulocytes from patients who did not have RA but were on NSAID therapy was investigated. With the five patients studied, there appeared to be a decrease but no significant differences were found between them and the other groups (see Figure 5.3). In view of these results, it was decided to study the response of granulocytes from volunteers before and after 72 hours treatment with an anti-inflammatory drug. Soluble aspirin was chosen as the agent, at 600 mg tds. The control group consisted of 9 members of staff, three of whom did not complete the three -day course because of side-effects. The last dose was taken two hours before bleeding in those completing the course. The details are given in Figure 5.4., and representative titres in Figure 5.5 (from three subjects who completed the therapy). It was obvious that there was no overall alteration consistent with the difference that had been found between RA patients and controls.

The release by PMN in response to 200 μg ml^{-1} HAGG after the subtraction of background release was compared to the IgM RF titre and to the Clq-binding activity of each patient's serum. Regression analysis was employed. For the R.F. comparison, r equalled -0.229, and with n = 22, this was not significant. When comparing Clq-binding activity with enzyme release, however, r equalled -0.751 ($n = 7$, $0.05 < P < 0.1$) i.e. possibly significant. Most of the patients had an ACA of less than 1 in 2.

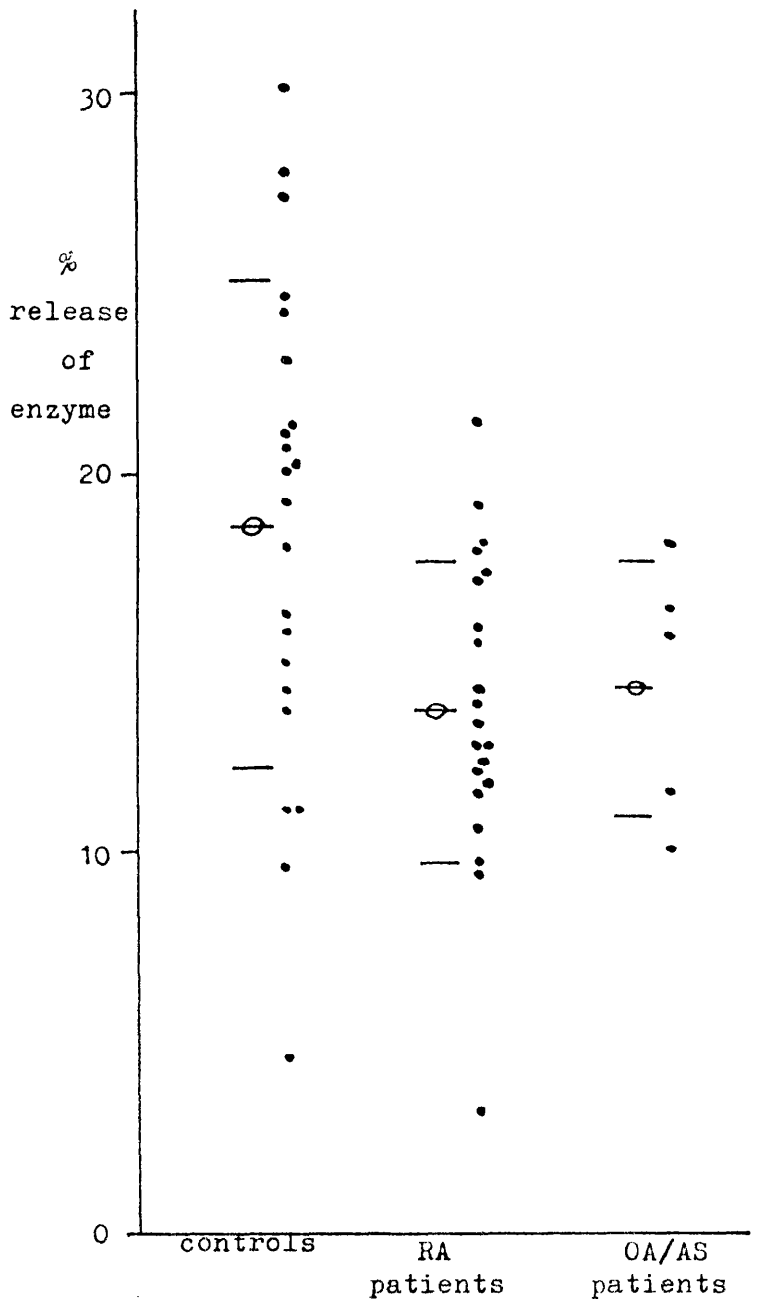

Figure 5.3

The release of β-glucuronidase in response to 200 μg ml^{-1} HAGG by patient and control groups.
⊖ represents the mean and bars (-) ± 1 SD.
Controls to RA patients .01> P> .001
All other pairs of variables not significant.

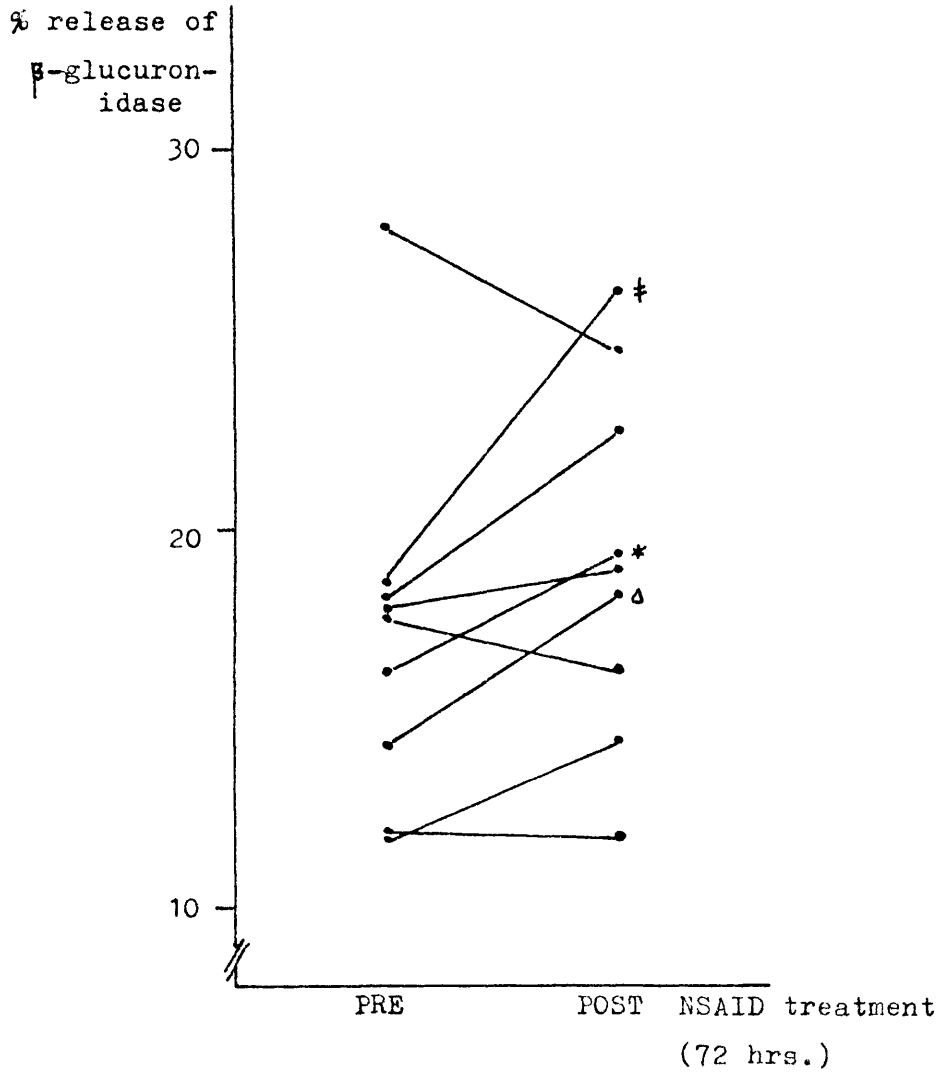

Figure 5.4

The effect of aspirin therapy on the responsivness of granulocytes to 200 $\mu g\ ml^{-1}$ HAGG.

The administration ceased at 24 hours ≠
36 hours *
60 hours Δ

See also Figure 5, for varying concentrations of aggregate.

Figure 5.5.

The effect of aspirin therapy on the responsiveness of granulocytes to aggregated IgG.

Blood samples were taken before (Δ) and after (o) 72 hours of aspirin administration, and each cell preparation was exposed to increasing concentrations of HAGG.

The data are from three of the normals shown in Figure 5.4.

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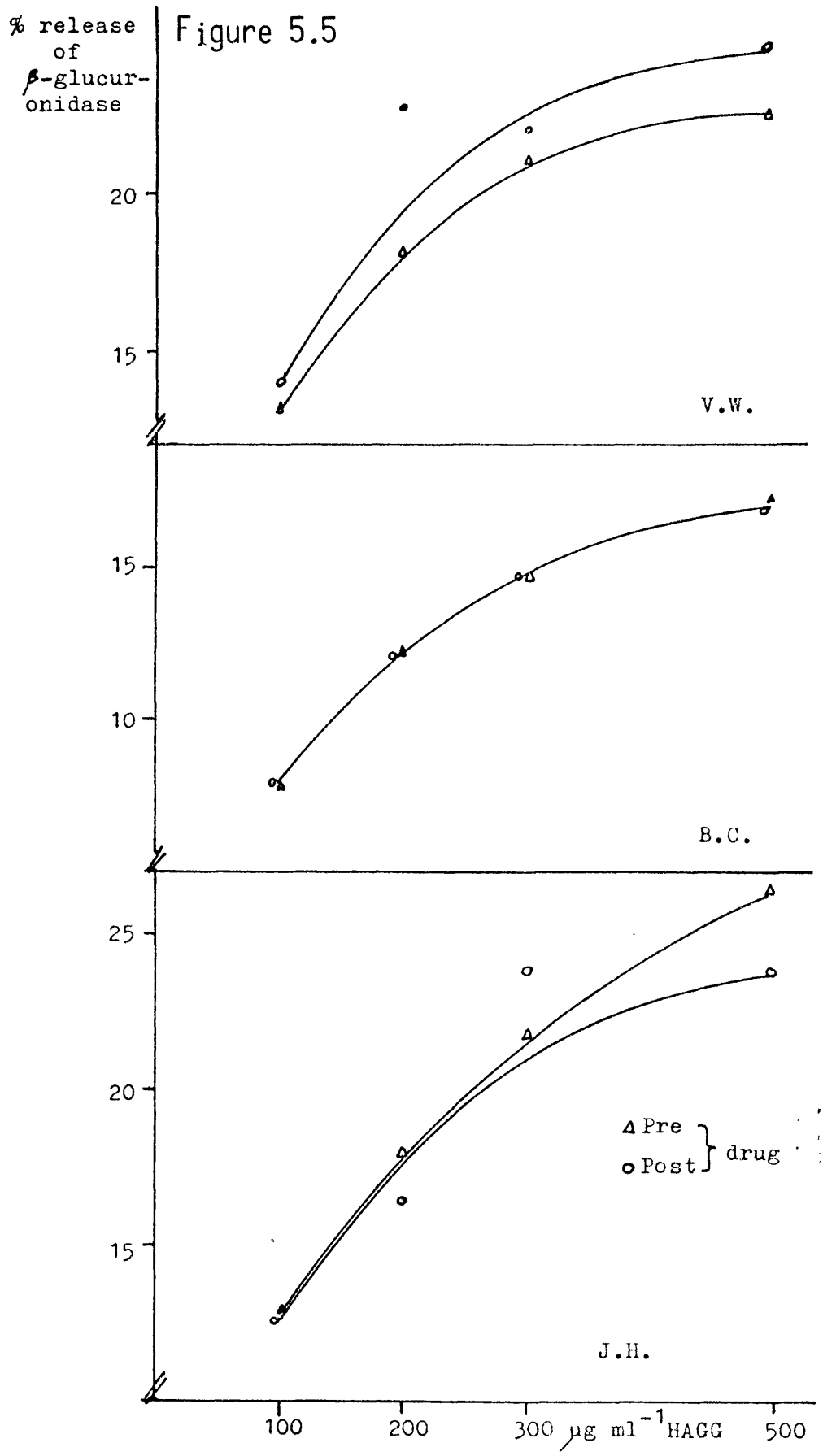
Figure 5.5


% release of β-glucuronidase
20
15
V.W.
15
10
B.C.
25
20
15
Δ Pre
o Post
drug
J.H.
100
200
300 μg ml⁻¹ HAGG
500

Synovial fluid granulocytes.

The response of granulocytes from paired peripheral blood and synovial fluid was investigated in a group of patients with active RA and effusions of the knee. The synovial fluid PMN had a viability of between 91 and 99% after they had been isolated, compared with a greater than 99% viability by trypan blue exclusion for their counterparts from the blood. The percentage LDH release values were 2.4 to 9.6 and 1.0 to 6.2 respectively. Hyaluronidase treatment did not decrease the viability of the cells (1.0% compared to 3.3% for untreated PMN, one patient).

Each cell type was exposed to varying concentrations of HAGG and the difference in response between the blood and fluid cells was determined. The results are shown in Table 5.1: there were no consistent differences between peripheral blood and synovial fluid PMN. The changes did not correlate with the drug treatment of the patients, nor with their serum IgM RF titre. Clq-binding estimations of blood and fluids were not carried out. The hyaluronidase treatment of peripheral blood granulocytes decreased their response to lower levels of aggregate but not to the same extent as was found for the fluid leucocytes. Viability was not decreased (Table 5.1).

Discussion.

Of the cells studied in these degranulation experiments, it was the granulocytes from blood that in the RA group as a whole reacted differently to the normals in that they released less % β-glucuronidase than did control cells. In this case, the reactivity may be related

Table 5.1. The release of enzyme by paired peripheral blood (PB) and synovial fluid (SF) granulocytes in response to aggregated IgG. β-glucuronidase release is expressed as the change in % output above background levels.

Abbreviations:

ND	not done
LDH	lactic dehydrogenase
RF	IgM rheumatoid factor titre, by direct antiglobulin test
%V SF	% viable cells in synovial fluid granulocytes by trypan blue exclusion. PB cells were all at least 99% viable.
NSAID	non-steroidal anti-inflammatory drugs
HPB	hyaluronidase-treated PB granulocytes

decreased release ↓ increased ↑ no change ↕

Patient	Release of β-glucuronidase with HAGG, μg ml^{-1}			% LDH	%V	R.F.	Treatment
	200	300	500	release	SF	$Titre^{-1}$	
G.S. PB	20.3	19.2	20.2	N.D.	99	neg	NSAID
SF	10.6 ↓	16.9 ↓	12.6 ↓				
C.T. PB	15.7 ↑	16.9 ↑	16.9 ↑	N.D.	99	neg	NSAID
SF	19.9	22.9	17.5				
L.B. PB	18.7	ND	ND	N.D.	99	neg	NSAID
SF	5.4 ↓						
G.M. PB	11.0 ↑	14.8 ↑	13.0 ↑	N.D.	99	neg	NSAID, D-penicillamine
SF	12.6	15.0 ↓	19.0				
J.B. PB	20.7 ↑	24.4	ND	1.6-2.2	95	16	NSAID, lithium
SF	20.2 ↓	20.4 ↓		2.4-6.4			
S.O. PB	10.1 ↑	13.1 ↑	16.0 ↑	2.5	91	32	NSAID, withdrawal from
SF	16.1	17.5	19.6	9.6			gold
R.P. PB	9.2 ↑	9.8	9.8 ↑	6.0		64	NSAID
SF	9.8 ↓	8.0 ↓	9.9 ↓	3.2	98		
E.H. PB	14.9 ↑	ND	16.9	ND		64	NSAID
SF	15.6 ↓		12.3 ↓		99		
E.C. PB	18.9	22.2	16.4	3.3		64	NSAID
SF	13.5 ↓	13.0 ↓	9.8 ↓	9.1	98		
HPB	16.0 ↓	15.9 ↓	16.2 ↕	1.0			
A.G. PB	15.1	20.6	21.0	2.8		256	NSAID, methylprednisolone,
SF	9.3 ↓	10.3 ↓	9.6 ↓	6.7	93		cyclophosphamide

to the Clq-binding activity in the sera of the patients. Although chemokinesis of peripheral blood neutrophils is not apparently affected by the serum immune complexes (Kemp *et al.*, 1980), the uptake of sensitized sheep red blood cells by human monocytes is definitely inhibited by inclusion of immune complex-rich serum in the *in vitro* incubation (Temple and Loewi, 1977). The possibility of phagocytosis of immune complexes occurring in the blood stream is much less than that in the tissues because granulocytes need a surface on which to crawl. Indeed, one study failed to demonstrate intracytoplasmic inclusions of IgG, IgM or C3 in blood leucocytes from seropositive RA patients, while showing their presence in SF leucocytes (Cats, Lafeber and Klein, 1975). Vaughan discovered inclusions in the SF leucocytes of 15 RA patients, six of whom also exhibited them in their PB leucocytes, though less strongly than those from the fluid (Vaughan *et al.*, 1968). Immunofluorescence was used for both studies. In the absence of paired immunofluorescence and degranulation data, it is impossible to tell whether the presence of immune complexes has any bearing on the ability of the phagocytes to react to the IgG aggregate.

The studies that have found similar behaviour of normal and patients' PMN have involved complete uptake of latex (Hallgren, Hakansson and Venge, 1978) and yeast (Turner, Schumacher and Myers, 1973) rather than the frustrated phagocytosis employed in the system described in this chapter. This may be an important variance.

The diminished ability of patients' granulocytes to respond to the phagocytic stimulus did not appear to be related to either

age or drug therapy. Herzer and Lemmel (1980) have shown that NSAID only inhibit phagocytosis of latex and cell activation by dextran sulphate when the drug is present in the incubation medium, and that at a concentration five times that of *in vivo* levels. Pre-incubation of the cells followed by washing abrogated this effect. Kemp noted that the ability of neutrophils from peripheral blood to respond to a chemotaxin was in fact increased in RA patients taking aspirin compared to a group who were not doing so (Kemp *et al.*, 1980). In the same study, he ascertained that the degree of chemokinesis could not be correlated with serum immune complexes as measured by Clq-binding as mentioned above. However, synovial fluid PMN chemokinesis was depressed to an extent inversely related to the joint fluid complex level. In the study described here, there was no consistent difference in the reacitivity of fluid PMN towards the phagocytic stimulus as compared to that of peripheral blood granulocytes. The slightly increased LDH release agrees with the findings of Van de Stadt and colleagues (1980).

If there is an inherent defect in the granulocytes of RA patients, it is not manifest in the circulating monocytes. The results suggest that there is no difference in the phagocytic response that leads to degranulation between those cells from patients with RA and control volunteers. This agrees with the findings of Bar-Eli and colleagues (1980) who did, however, demonstrate a decreased bactericidal activity in patients' cells, a property not tested in this study. Fc-mediated phagocytosis has been shown to be the same in RA's and normals' monocytes by Hurst and Nuki (1981). The similarity of response may reflect a genuine identity

with normal mononuclear phagocytes, or the fact that since the circulating cell is not fully developed in its secretory properties (Bennet and Cohn, 1966) the difference has not yet become evident. This question might have been clarified by incubating peripheral blood monocytes for 24 to 72 hours in the presence of aggregate, with regular sampling for β-glucuronidase release. Alternatively, they could be cultured in its absence and subsequently challenged with the stimulus.

Degranulation is only onepart of the phagocytic process (see Chapter 1, including Figures 1.2 and 1.3). In view of the differences between cells noted in the experiments detailed here, some other aspects were also studied. The findings are presented in Chapters 7 and 8 of this thesis.

CHAPTER SIX THE MODULATION OF PHAGOCYTOSIS

Introduction

The degree of phagocytosis of particles can be altered by the presence of molecules such as complement, rheumatoid factors and immune complexes.

The opsonization of particles such as zymosan with the complement component C3 greatly enhances their ability to stimulate phagocytes, more so than does coating with just IgG (van de Stadt, van de Voorde-Vissers and Feltkamp-Vroom, 1980). When C3 interacts with IgG or IgM-containing immune complexes (anti-DNA/DNA), their growth into large complexes is inhibited, but when the complexes contain only IgM anti-DNA the presence of C3 is necessary for their processing by monocytes (PMN processing is absent). The same researcher has reported that the molecule Clq increases the size of small IgG anti-DNA/DNA complexes, but has no detectable effect on the IgM-complexes (Lamers, 1980). In contrast, it seems that Cl is able to inhibit IgM RF - mediated agglutination of IgG-coated latex and even distintegrate pre-formed agglutinates (Hallgren, 1980).

The effect of immune complexes on the phagocytosis of other particles is one which appears to be solely cell-dependent. The observation that their ingestion can diminish subsequent phagocytosis (Turner, Schumacher and Myers, 1973) has already been discussed in Chapter 5.

In terms of the behaviour of rheumatoid factors, any alteration in the ingestion of immune complexes by monocytes and granulocytes appears to be directly attributable to their shifting the equilibrium from soluble to insoluble complexes, and vice versa (Ward and Zvaifler, 1973b; Turner *et al.* 1976; Treadway *et al.*, 1979; Lamers, 1980; Lamers, de Groot and Roos, 1981). *In vitro*, IgM RF has been shown to increase the amount of free antigen in pre-formed immune complexes (Alkner and Hansson, 1979). However, RF has been reported to alter the degranulation of neutrophils in response to surface-bound aggregated IgG (Timms, Johnson and Henson, 1975), a process which is unlikely to involve equilibrium effects. The authors stressed its complement independence, and its possible importance in the role of IgM RF as a protective agent in rheumatoid arthritis (Timms, Johnson and Henson, 1975).

These three variables were investigated, making use of the degranulation in response to aggregated IgG that has been described in previous chapters.

Materials and Methods

The granulocytes and monocytes were isolated from peripheral citrated blood by the method detailed in Chapter 3 (see Figure 3.3). Monocyte-enriched populations were used for the rheumatoid factor experiments, and mononuclear cells for all others. The details of the purification and aggregation of IgG; the isolation of rheumatoid factors and of putative complexes from synovial

fluids and the preparation of anti-immunoglobulin allotype reagents have been laid out in Chapter 2, section 5. The plasma sample D.C. that contained complexes was the same as that used in Chapter 4. All normal sera used were ACA negative and had normal levels of CH_{50}, C3 and C4.

1. Complement experiments:

Normal serum was decomplemented either by heat inactivation at 56°C for 30 minutes or by inulin streatment, as used by Lamers (1980). Briefly, inulin from Dahlia tubers (Sigma) was washed twice in distilled water (5000 g 10 mins). Normal human serum was incubated for two hours at 37°C with the washed inulin at 50 mg ml^{-1}, then the latter was removed by centrifugation for 20 minutes at 5000 g. The CH_{50} after these treatments was negligible, being less than 10 units compared to the 350U found in untreated serum. Complement coating was carried out by incubating the aggregated IgG with 2.5, 5 or 10% normal serum for one hour at 37°C followed by storage in crushed ice, for up to two hours, until required. The decomplemented sera were used as control incubations. The coating with 10% serum, for instance, became a 0.25% serum concentration when the aggregate was added to the cells. The aggregate concentration was 100 µg ml^{-1}, PMN were at 5 x 10^6 ml^{-1} and MNC at 1.25 x 10^6 ml^{-1} (monocytes). The cells were from normal controls for these experiments. They were resuspended in M 199 but FCS was not added.

2. Complex experiments:

Pre-incubation of phagocytes was carried out in the complex-containing plasma D.C. vol/vol with M 199, or in M 199 plus 10% FCS, for 45 minutes at 37°C on a rocking table. Each tube was centrifuged at 150 g for 10 minutes and the supernatants discarded; the pelleted cells were washed once in PBS. The appropriate stimulant (medium or HAGG at 200 μg ml^{-1}) was added to the cell pellet in 250 μl of medium and incubated for a further 45 minutes at 37°C. Centrifugation at 1000 g for 10 minutes followed this, and the supernatant was harvested. The pellet was lysed with BSA/Triton and centrifuged (see Chapter 2). The viability of the cells was estimated by trypan blue. Two subjects (E.C. and A.F.) were rheumatoid patients and two were normal controls (J.W. and P.S.).

3. Rheumatoid factor experiments:

The phagocytosis assays were identical to that described in Chapter 2, save for the addition of the appropriate concentration of purified rheumatoid factor and correspondingly less tissue culture medium. Two preparations of rheumatoid factor were used; a purified IgM RF kindly donated through Dr. P.M. Johnson, University of Liverpool and a mixed IgM-IgG RF that was given by Dr. C. Elson, University of Bristol. The latter preparation contained 29% IgM RF and 71% IgG RF, as measured by radio-immunoassay, and was given the abbreviation MxRF (mixed rheumatoid factor). The IgM RF had a protein concentration of 700 μg ml^{-1} with a latex titre of 1/40; the MxRF at 2.5 mg ml^{-1} titred to 1/2560 (equivalent to 1/160 at 700 μg ml^{-1}). Monocytes were used at 1.25 x 10^{6} ml^{-1}.

Results

4. Complement experiments:

When fresh or heat-inactivated sera were allowed to react with HAGG prior to the addition of the cells, an alteration in the cellular response was seen when compared to HAGG in buffer alone (Table 6.1). This change was very dependent upon the person, but in some increased slightly with increasing fresh serum concentration, but less so with heat-inactivated serum. In some subjects (J.F. and S.W.) the monocytic activity seemed to be inhibited by fresh and HI serum while in others (R.E. and A.R.) it seemed to be stimulated inboth. Decomplementation with inulin did not alter the level of response of PMN and the single result with monocytes suggested the same. These experiments showed that under the conditions used, the fixation of complement by HAGG had no unipolar effect on degranulation by monocytes or by granulocytes.

In the absence of heat-inactivated FCS, the release by PMN was less than in its presence (Table 6.2), and this reflects the result found with human serum, despite the differences in final serum concentration. This foetal calf serum had no demonstrable CH_{50} activity.

5. Experiments with complexes:

The viability of both monocytes and granulocytes was not affected by incubation in plasma D.C. v/v M 199 or M 199 with 10% FCS (viability was at least 99%), and this did not decrease with a second incubation. Despite this, the background release from the cells was higher than usual, whether preincubation had taken

	Serum					
	2.5%F	5%F	10%F	2.5%HI	5%HI	10%HI
MNC						
R.E.	1.23	2.32	1.59	2.86	2.73	2.68
A.R.	1.86	1.18	0.98	2.16	1.95	2.05
J.F.	0.56	0.80	0.78	1.05	0.88	1.07
S.W.	0.60	0.43	0.82	0.63	0.74	0.64
PMN						
R.E.	1.04	1.12	1.59	0.98	0.70	1.11
A.R.	0.70	1.15	1.04	0.79	0.92	0.96
J.F.	1.04	1.13	1.50	1.32	1.18	1.48
S.W.	1.00	1.10	1.10	0.94	1.02	0.65

		Serum		
		25%F	25%HI	25%I
MNC	M.K.	1.14	1.64	1.10
PMN	M.K.	1.37	1.28	1.19
	S.F.	1.33	1.31	1.27

L.D.H. values; MNC zero - 11.3% PMN 0.7 - 13%

Table 6.1. The effect of coating aggregated IgG with serum treated in different ways.

Key: F Fresh human serum HI heat-inactivated serum

I inulin-treated serum

Each value is the mean of duplicate tests, calculated using the following equation:

$$\text{release } (\beta\text{-glucuronidase}) = \frac{\text{release HAGG+additive - additive alone}}{\text{release HAGG+buffer - buffer alone}}$$

	HAGG $\mu g\ ml^{-1}$		
	50	200	500
D.J.			
+FCS	8.9	17.7	25.8
-FCS	6.3	10.7	16.0
B.C.			
+FCS	6.3	18.1	23.3
-FCS	5.2	10.6	14.5

Table 6.2. The effect of 10% heat-inactivated foetal calf serum on the release of β-glucuronidase by PMN.

place in medium or plasma (9.0 ± 5.5 (n = 8) for double incubation, 3.7 ± 2.9 (n = 8) for the single). Allowing for this (Table 6.3), the response of preincubated cells was in all but one test (E.C. PMN) less than that from cells treated only once (that is, no pre-incubation). Where the phagocytes had been exposed to plasma D.C. rather than to medium, they were less able to respond to 200 μg ml^{-1} HAGG, in 3 out of 3 MCP and 2 of 4 PMN preparations.

6. Experiments with rheumatoid factors:

Monocyte-enriched and granulocyte preparations were allowed to react with aggregated IgG with and without various concentrations of the MxRF. The MxRF alone induced no significant degranulation, being on average 0.8% for MEP and 1.2% for PMN. The results were expressed as the release above background of β-glucuronidase in the presence of HAGG plus the RF, divided by that in the presence of HAGG alone. A figure of less than unity, therefore, represents inhibition, and more than one describes stimulation of degranulation by the RF. It can be seen (Figure 6.1) that an increasing concentration of MxRF caused a progressively greater inhibition of degranulation by monocytes, but not by PMN. These latter cells were, if anything, slightly stimulated. Patient and control cells behaved comparably, so the data for the two groups was pooled.

When a similar experiment was carried out using the pure IgM RF, a different pattern of behaviour was seen (Figure 6.2). This protein had a minor inhibitory effect on monocytes, even at 50 μg ml^{-1}, while at the same concentration the MxRF was very active. The difference between the two was significant,

	Mononuclear cells			Granulocytes		
	M199 + 10%FCS	HAGG	Δ	M199 + 10%FCS	HAGG	Δ
One incubation:						
E.C.	1.5	12.8	11.3	1.4	4.7	3.3
J.W.	3.8	17.0	13.2	6.1	10.7	4.6
A.F.	8.7	16.2	7.5	1.9	13.6	11.7
P.S.	5.7	12.5	6.8	0.6	10.1	9.5
Pre-incubation with M 199 + 10% FCS						
E.C.	13.4	20.3	6.9	3.5	9.0	5.5
J.W.	20.5	30.5	10.0	6.9	6.7	-0.2
A.F.	9.5	14.5	5.0	3.8	13.1	9.3
P.S.	6.3	ND	ND	3.7	7.6	3.9
Pre-incubation in D.C. plasma v/v M 199						
E.C.	13.3	16.3	3.0	8.6	9.2	0.6
J.W.	20.1	18.6	-1.5	11.1	11.8	0.7
A.F.	7.3	9.6	2.3	4.7	10.3	5.6
P.S.	7.8	12.8	5.0	3.6	9.3	5.7

Table 6.3 The effect of pre-incubating cells in a plasma with a high Clq-binding (D.C. plasma), or in M 199 plus 10% Foetal calf serum, prior to challenging them with 200 $\mu g\ ml^{-1}$ of HAGG.

Each value is the mean of duplicate tests, and is % β-glucuronidase released.

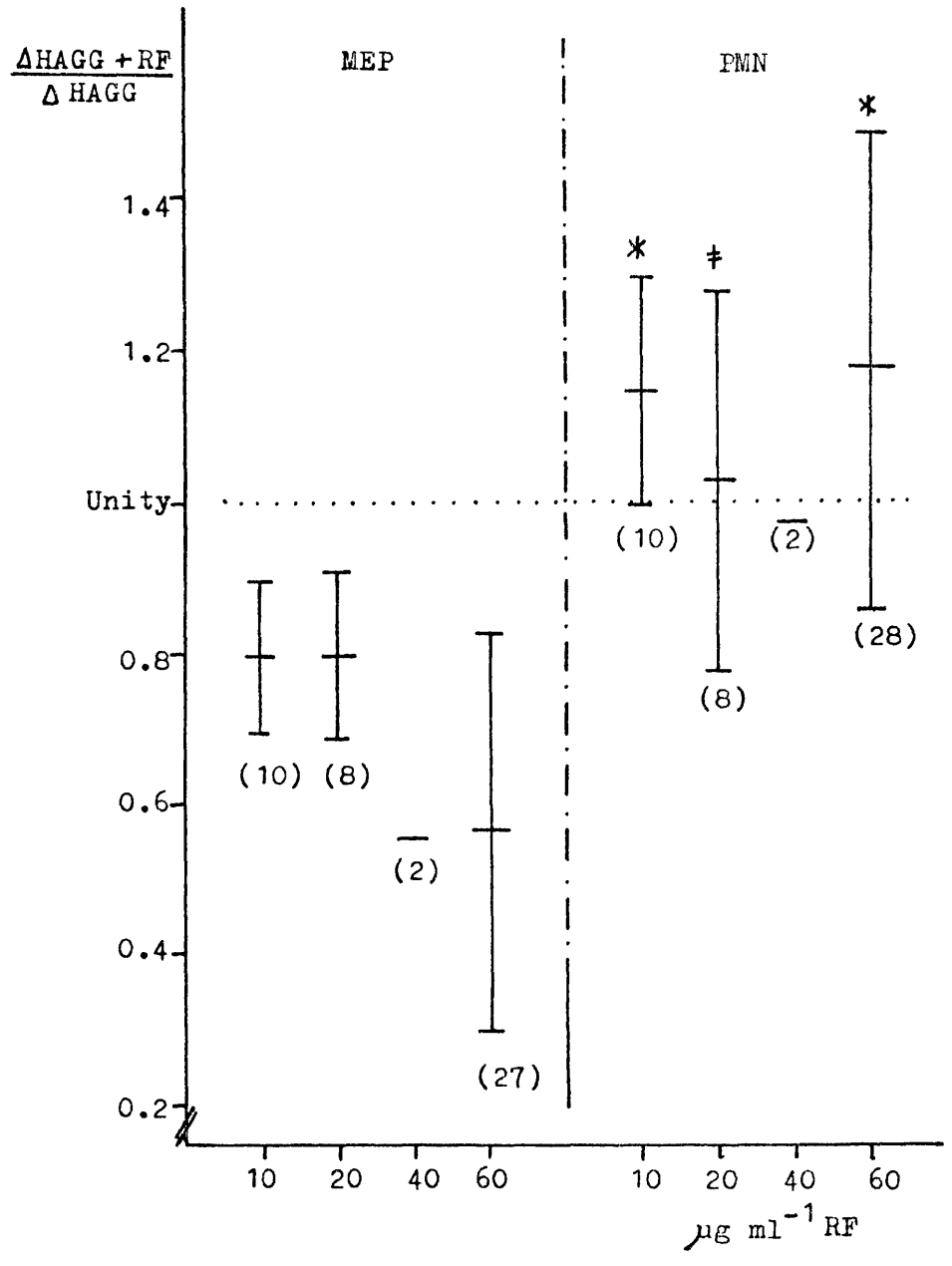


Figure 6.1

The effect of rheumatoid factor on the response to HAGG.

Results are expressed as $\bar{x} \pm 1$ SD, with the number of tests in parentheses.

The RF used was mixed IgG/IgM, see text, and HAGG was at 200 μg ml^{-1}.

Statistical differences between PMN and MEP

*P < .001

ǂ .02 < P < .05

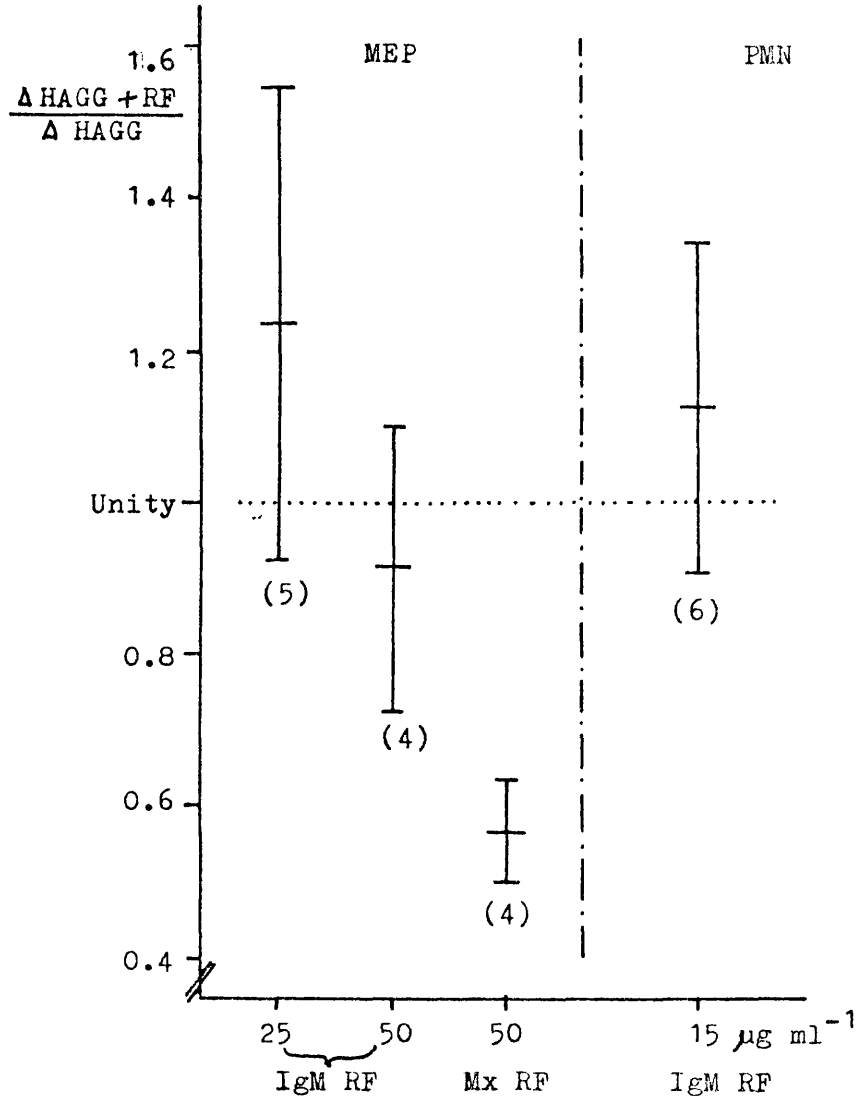


Figure 6.2

The effect of different rheumatoid factor preparations on the response to HAGG.

Results are expressed as $\bar{x} \pm 1$ SD, with the number of tests in parentheses.

The RF used was purified IgM RF or mixed IgG/IgM RF (MxRF), and HAGG was at 200 $\mu g\ ml^{-1}$.

Difference between 25 and 50 $\mu g\ ml^{-1}$ IgM RF was NS.

Difference between IgM RF and MxRF, 50 $\mu g\ ml^{-1}$, $.02 < P < .05$

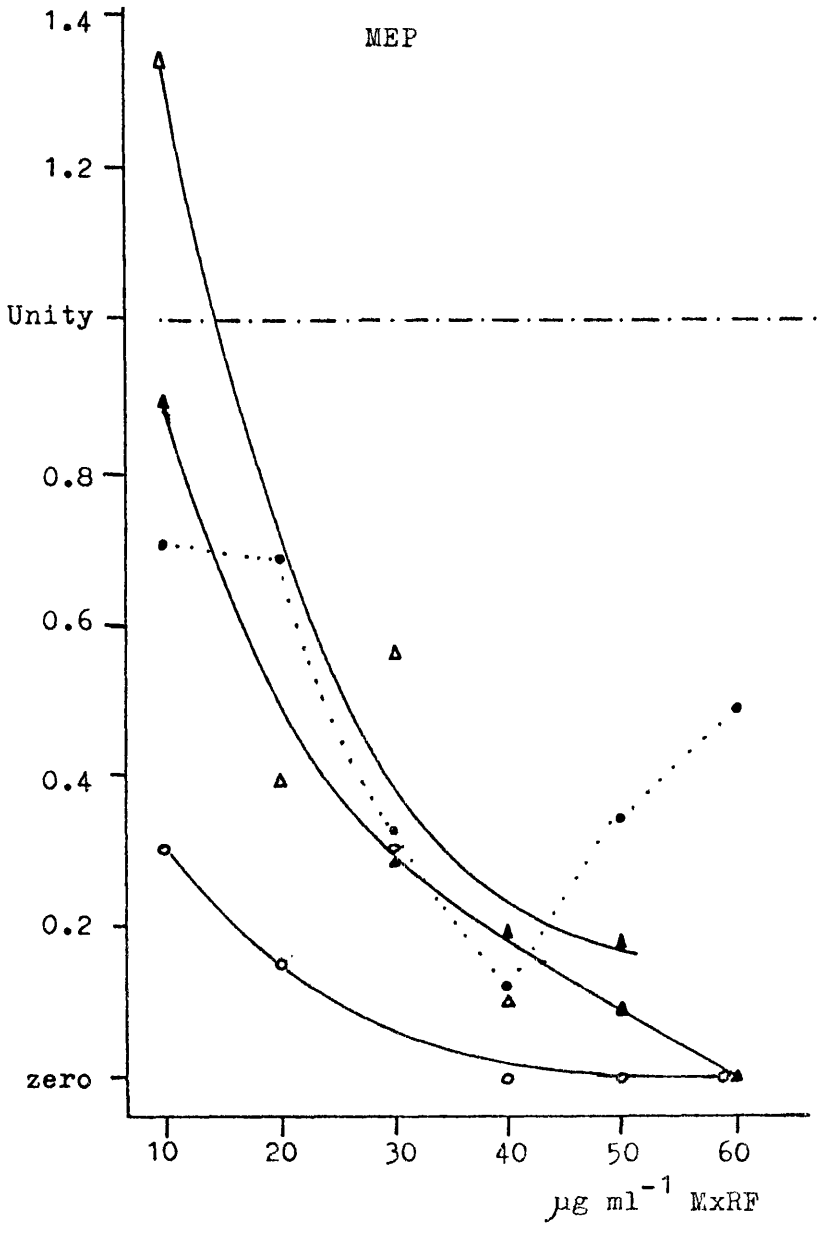


Figure 6.3

The effect of altering both rheumatoid factor and aggregate concentrations.

- Δ——Δ B.M. HAGG at 50 µg ml^{-1}
- ▲——▲ B.M. HAGG at 200 µg ml^{-1}
- o——o S.W. HAGG at 50 µg ml^{-1}
- •·····• S.W. HAGG at 200 µg ml^{-1}

Zero represents the maximum possible inhibition.

0.05 > P >0.02. Neither preparation affected granulocyte function to any degree.

Both RF (mixed RF) and aggregate concentrations were altered and some of the results for monocytic cells are shown in Figure 6.3. In three cases the inhibition increased with MxRF concentration whatever the aggregate concentration. However with S.W. cells in response to 200 μg ml^{-1} HAGG there was a progressive inhibition from 10 to 40 μg ml^{-1} of the rheumatoid factor followed somewhat unexpectedly by a rise in degranulation (see dotted line, Figure 6.3). This suggested that maximal inhibition occurred at 40 μg ml^{-1} of MxRF. The behaviour of the polymorphs was not affected by changing the concentration of the aggregate (results not shown). The mean LDH release fro the monocytes was 6.5% and for granulocytes 2.0%.

The pure IgM was again inactive when a chequerboard was tried (not shown). The mean LDH release for monocytes was 2.5% and for PMN was 5.5%.

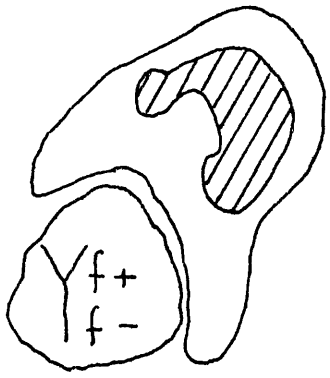
Discussion

The effect of the rheumatoid factor was difficult to interpret because of the probable presence of self-associating complexes in the mixed rheumatoid factor preparation. The following experiment was designed to clarify the position. The allotypic marker Glm (f) lies at amino acid position 214 in the hinge of the IgG1 molecule (van Loghem, 1978) and IgM antibody activity towards it is found

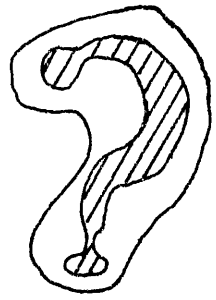
in a small number of Glm (f)-negative people. The Blood Transfusion Service (Southmead) were kind enough to donate some anti-Glm (f), and some Glm (f)-positive serum, and details of their purification are given in Chapter 2. The possible reactions between such an antibody and aggregated or free IgG1 are shown in Figure 6.4. The phagocyte would be expected to respond identically to aggregated IgG1 of either allotype, but upon the addition of the IgM fraction that contained anti-Glm (f) a difference in behaviour might occur between the two. Whereas the antibody would not react with Glm(f)-negative IgG, it would bind to the positive; it might mask antigenic sites on the IgG, or create new ones at the Fc end of the IgM. If free Glm (f)-positive IgG were to be added, then soluble complexes might form with the antibody and be taken up by the phagocytes. The degranulation response towards aggregate might then be diminished. The use of Glm (f)- negative aggregate in this case abrogates the chance of the antibody reacting with it, and so increases the specificity.

These experiments would be directly comparable to the use of pure IgM RF and self-associated IgG/IgM RF with the added specificity gained by having an aggregate with which the antiserum cannot react. Unfortunately, it was not possible to bring these ideas to fruition because of the technical problems outlined below.

Purified Glm(f)-positive IgG1 from normal serum or from a myeloma (Mrs W) did not aggregate to the same degree as did the IgG^1 used in previous experiments (Mrs H) which itself typed as heterozygous, the normal IgG being positive and the myeloma negative. The degree to which myeloma γ-globulins aggregate on exp-

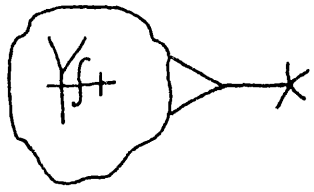


Degranulation in response to aggregated IgG1, G1m(f) positive or negative.

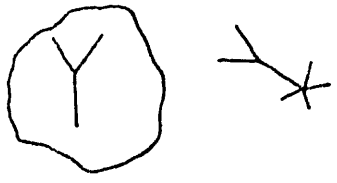


Addition of anti-G1m(f)

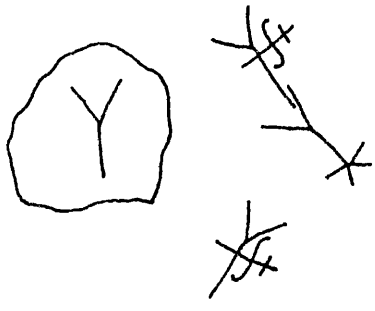
Possible effect



Direct



None

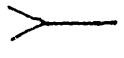


Indirect

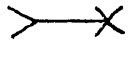
Figure 6.4

A scheme illustrating the use of immunoglobulin allotypes towards an understanding of the action of rheumatoid factor.

Code:

 G1m(f) negative IgG

 G1m(f) positive IgG

 anti-G1m(f) IgM

osure to heat has been discussed in Chapter 2, Section 5 (vii). A Glm(f)-negative myeloma could not be found in the few locally available. The antiserum had a titre of 1/4 by haemagglutination prior to isolation of the IgM fraction when it dropped to ½, at 2.5 mg ml^{-1}. This activity was considered too low for definite results. Anti-allotypic sera of high titre are less readily available because of their use as routine reagents.

Owing to these problems, no insight could be gained into the modulating effects of RFs on phagocytosis by the use of allotypes. Two types of rheumatoid factor were studied with respect to their ability to modulate the activity of phagocytes. A preparation that consisted of pure IgM RF had little effect on either monocytes or neutrophils (see Figure 6.2). In oontrast, the preparation that contained both IgG RF and IgM RF had a dose-dependent inhibitory activity upon monocytes (but none on granulocytes), as shown in Figures 6.1 and 6.2. These results with pure IgM RF and the mixed IgG/IgM RF suggest that the presence of the IgG RF mediates the change in IgM activity from having no effect on phagocytosis by monocytes to an inhibitory one. *In vivo*, this inhibition could lead to increased circulating complexes. Recently, Scott and colleagues (Scott D.G.I. *et al.*, 1981) noted that RA patients with vasculitis had a higher IgG RF level than did those with synovitis and also were more often ACA positive. The anti-complementary activity of serum correlates with the presence of immune complexes that contain IgG as the antibody and this is thought to be because IgM antibodies are somewhat labile, and this assay for complexes involves an incubation step of one hour at

56°C (Johnson and Mowbray, 1977). This anti-complementary activity correlates too with the presence of vasculitis in pathological states, and within rheumatoid arthritis is partly characteristic of vasculitis. It is possible therefore that the presence of the circulating, complement-binding complexes is directly related to the presence of IgGRF(in addition to the IgM type), and this could be explained by the experimental findings that cells destined to become residents of the tissue mononuclear phagocyte system are made refractory towards aggregated IgG when both RF types are present.

Onyewotu and associates (1975) found that the presence of an excess of RF enhanced the uptake of soluble aggregates by guinea pig macrophages, but produced an inhibition when the aggregate predominated. The sera from vasculitic patients contained immune complexes that enhanced uptake of the soluble aggregate; the authors proposed that free valancies on the RF in the complexes would take up the soluble aggregate and present it as a larger molecule to the cell. This would only occur if the RF already had some antigen binding sites full, for example in circulating immune complexes in vasculitis.

The phenomenon of enhancement-inhibition was not seen when the mixed rheumatoid factor was used in the present experiments. Inhibition increased with the RF concentration (Figure 6.1). The purified IgM RF appeared to behave in a way opposite to Onyewotu's reagent, but not definitively (Figure 6.2). The difference in behaviour is most likely to be due to the varying size of the aggregates: the enlarging and precipitating effect of RF on

soluble aggregates (with PMN, Turner *et al.*, 1976 and with both PMN and monocytes, Lamers, 1980; 1981) would be negligible on a particulate aggregate.

The lack of effect on neutrophils would not be surprising, per se, since they do not express IgM receptors (Henson, 1977). However IgM RF has been shown to inhibit the release of β-glucuronidase from PMN responding to surface-bound aggregated IgG (Timms, Johnson and Henson, 1975) and to precipitated BSA/anti-BSA complexes (Ward and Zvaifler, 1973b) and this protective effect may be mediated by covering the stimulant with a protein that the neutrophil cannot bind (IgM). This change was not demonstrable using either the pure IgM RF or the mixed preparation (Figures 6.1 and 6.2), although it is possible that a higher concentration of the IgM RF might have produced such an alteration. Lack of this reagent precluded further experiments being performed.

The interaction of neutrophils and aggregated IgG or precipitated BSA/anti-BSA complexes is thought to be complement independent, and this has been demonstrated in this study (Timms, Johnson and Henson, 1975; Ward and Zvaifler, 1973a and 1973b). Depletion of serum C1/C2 by heating or C3 by inulin treatment had no effect on the cellular response. If serum (either fresh or inactivated) was omitted, however, the cells responded less well (Tables 6.1 and 6.2). Which serum component(s) might be responsible for this was not investigated.

Previous ingestion of immune complexes can decrease the

subsequent phagocytosis of yeast particles (Turner, Schumacher and Myers, 1973), even if the two stimuli are present together (Svensson, 1980). The same worker has also demonstrated that sera containing immune complexes can also do this, and this was demonstrated with the plasma D.C. The higher background release after two incubation steps made interpretation difficult (Table 6.3), but preincubation in the plasma certainly seemed to have an inhibitory effect. The influence of immune complexes on subsequent phagocytosis has already been discussed in the previous chapter. The increased background secretion of β-glucuronidase was not apparently due to cell death (as assessed by trypan blue exclusion) although in the case of J.W. both cell types failed to respond to the challenge with HAGG, suggesting that they were not too healthy. LDH estimations were not carried out on this experimental group.

The action of these complexes and the rheumatoid factors needs to be investigated at a level that will measure the interaction of cells and stimuli without the need for degranulation. The mixed rheumatoid factor preparation may be exerting an influence because of the presence of self-associated immunoglobulin (complexes) a process that cannot occur in the pure IgM RF because no IgG is present. Such complexes might be expected to cause metabolic stimulation of the cells, a phenomenon independent of exocytosis (Henson and Oades, 1975; Kitagawa, Takaku and Sakamoto, 1980b; McPhail, Henson and Johnson, 1981). This possibility is investigated in a later series of experiments which are presented in Chapter Eight.

CHAPTER SEVEN FURTHER STUDIES ON PHAGOCYTES: I. SURFACE RECEPTORS

Introduction

Phagocytic cells respond to a stimulus if they can recognise it, and this is brought about through receptors in the plasma membrane. Neutrophils express receptors for chemotactic peptides (as previously indicated in Chapter 1 "Surface receptors on neutrophils") but possession of such receptors is not confined to the phagocytes. For instance chemotaxis is a property of very many non-fixed cells including bacteria and is obligatory for the formation of a fruiting body in the cellular slime mould *Dictyostelium discoideum* (Ashworth, 1973). Lymphocytes migrate to various parts of the lymphoid system in the presence or absence of antigen (Inchley *et al.*, 1976; Schlesinger, 1977), a property that is affected by treating the cell surface with enzyme. They possess different receptors according to the type of lymphocyte that they are; for instance, 'B' cells can bind the Fc region of IgG, and C3b (McConnell, 1976a). Mononuclear phagocytes and neutrophils also possess these two receptor types (Ehlenberger and Nussenweig, 1977; Scott C.,1981), whereby they are able to bind to immune complexes, an ability which has been harnessed by immunologists. The association constants for the interaction of macrophages with radiolabelled complexes have given an indication of the number, specificity and activity of surface receptors (Leslie, 1980). The surface distribution of Fc receptors on neutrophils has been studied by electron microscopy after soluble ferritin/anti-ferritin complexes have been bound (An, 1980). Yet another method utilizes fluorescein-labelled antibody towards the antigen that has been bound: recent experiments by Tenner and Cooper (1981) have used this technique to identify the range of cell types

that allow Clq binding.

Perhaps the most well established technique for receptor detection is that of rosette formation. When suitably sensitized erythrocytes are brought into close contact with the appropriate cell, rosettes of adherent red blood cells will form around the receptor-positive cells. This is equally applicable to cell suspensions (Henson, 1977), fixed sections (e.g. of bone marrow, Scott,C.,1981)or macrophage monolayers(Rhodes, 1977). The proportion of rosette-forming cells in a population is directly proportional to the degree of sensitization of the erythrocyte. The steepness of the sigmoid dose-response curve has been shown to alter with malignant disease, and with the state of activation of the mononuclear phagocyte (Rhodes, 1975 and 1977).

It was thought that an alteration in the Fc receptors on rheumatoid granulocytes might explain the difference found between them and the population from normal controls in their ability to respond to aggregated IgG (see Figure 5.1). This was investigated using a rosetting technique.

Patients and Methods

The patients chosen for the study had definite rheumatoid arthritis with active synovitis, and all were being treated with NSAID. These were 5 M and 7F, mean age 59 years (range 41 - 76), with a mean duration of disease of 4.3 years. For comparison, twelve healthy volunteers were also studied, 5 M and 7 F, mean age 41 years (range 21 - 62).

Rosette Assay

The IgG fraction used to sensitize calf erythrocytes was prepared from a rabbit antiserum raised against calf red cell ghosts, using affinity chromatography on Protein-A Sepharose Cl-4B (Pharmacia). The IgG was eluted with 1 M acetic acid and then dialysed back into a 10 mM phosphate, 150 mM NaCl-containing buffer, pH 7.4. This procedure was based on the method of Hjelm and Hjelm, as used by Hall (Hall, 1978). The IgG was stored at -20^{o}C in small aliquots and thawed only once before use.

Calf erythrocytes (Tissue Culture Services, Slough, Berks) were washed out of Alsevers solution into PBS containing $CaCl_2$ and $MgCl_2$, both at 0.01 g dl^{-1} (PBSCM). After three washes they were resuspended to 2% and sensitized with an equal volume of the diluted IgG preparation detailed above. The coating was carried out in a 37^{o}C water-bath for 30 minutes and the cells then washed thrice in PBSCM and resuspended to 1%.

Granulocytes were isolated from citrated peripheral blood using Percoll as described in Chapter 3 (Figure 3.3.), and re-suspended at 2 x 10^{6} ml^{-1} in PBS (without albumin) containing 0.16 g dl^{-1} glucose. Equal volumes (200 µl) of the two cell suspensions were mixed in polystyrene tubes (LP3, Luckams Ltd., Sussex), stoppered and incubated at 4^{o}C for 10 mins, followed by centrifugation at 20 g for 3 minutes, and a further incubation period of an hour at 4^{o}C. All tests were set up in duplicate.

Three drops of freshly filtered crystal violet solution

(about 30 mg per 50 mls PBS) were added to each tube in turn which was then mixed on a rotating turntable (20 revs. per min.) for one minute. A small amount of the resulting suspension was placed in a haemocytometer to scan for rosettes. A rosette was defined as three or more red cells surrounding, and in contact with, a leucocyte. At least one hundred white cells were counted for each test, and the percentage positive cells calculated.

Results

An initial experiment was carried out with normal subjects to ascertain the sensitivity of the rosetting assay when a final red cell concentration of 1% as well as the 0.5% was used. The IgG was titred in doubling dilutions from 1/200 to 1/1600, and unsensitized cells were always included. The results, which are shown in Table 7.1, demonstrate that although the percentage of rosetting cells was lower with the 0.5% red cells, so too was the level of false positives seen with the unsensitized cells. The size of the rosettes decreased in proportion to the degree of the erythrocyte coating, being very large at 1/200 and 1/400 and decreasing to 3 - 6 red cells at 1/1600. For the main study, a final erythrocyte concentration of 0.5% was chosen, and although unsensitized controls were set up in every case, the number of "E rosettes" was negligible.

The granulocytes from the groups of patients and controls were incubated with cells sensitized from 1/200 to 1/3200 dilutions of IgG. The results obtained are summarized in Figures

Subject	Final % RC	Coating on erythrocytes (Titre^{-1}) 200	400	800	1600	None
L.E.	1.0%	97	88	58	4	8
	0.5%	94	89	62	4	0
P.M.	1.0%	93	84	71	28	7
	0.5%	88	78	69	37	2
C.F.	1.0%	79	65	35	7	2
	0.5%	77	60	30	10	0
J.W.	1.0%	91	83	69	45	1
	0.5%	84	80	70	41	0

Table 7.1 The effect of altering both the final red cell (RC) concentration and the degree of coating with antibody in a standard rosette assay. For details see text.

7.1 and 7.2. There was no demonstrable difference between the two cell groups. When individuals were considered, the degree of rosetting was not related to the proportion of neutrophils in the granulocyte preparation; the mean percentage was 96 (range 91 - 99) compared to 3% eosinophils (range 1 - 9%). Occasional lymphocytes were present. Individual results could also not be related to the granulocyte count of the peripheral blood (results not given).

Discussion

This study indicated that there was no difference between Fc_{γ} receptor expression on the granulocytes of rheumatoid arthritis patients and normal controls. It has been noted that a leucocytosis can occur during an acute exacerbation of a chronic inflammatory process (Baum and Ziff, 1979), more leucocytes are released from the bone marrow, with the possibility that some are not quite mature . Hurst and Nuki's observation (1981) that patients with rheumatoid vasculitis show a defect in monocytic phagocytosis of complement-coated yeast is thought to be due to the presence of immature monocytes in the circulation (Hurst, personal communication). It has been shown (Scott C. 1979 and 1981) that Fc receptor expression increases during the phase between segmentation of the nucleus and release of the neutrophil into the circulation; increased emigration might therefore lead to an overall decrease in the ability to react with IgG-coated erythrocytes. Alternatively, the binding of immune complexes to the cell surface *in vivo* might mask the receptors needed for

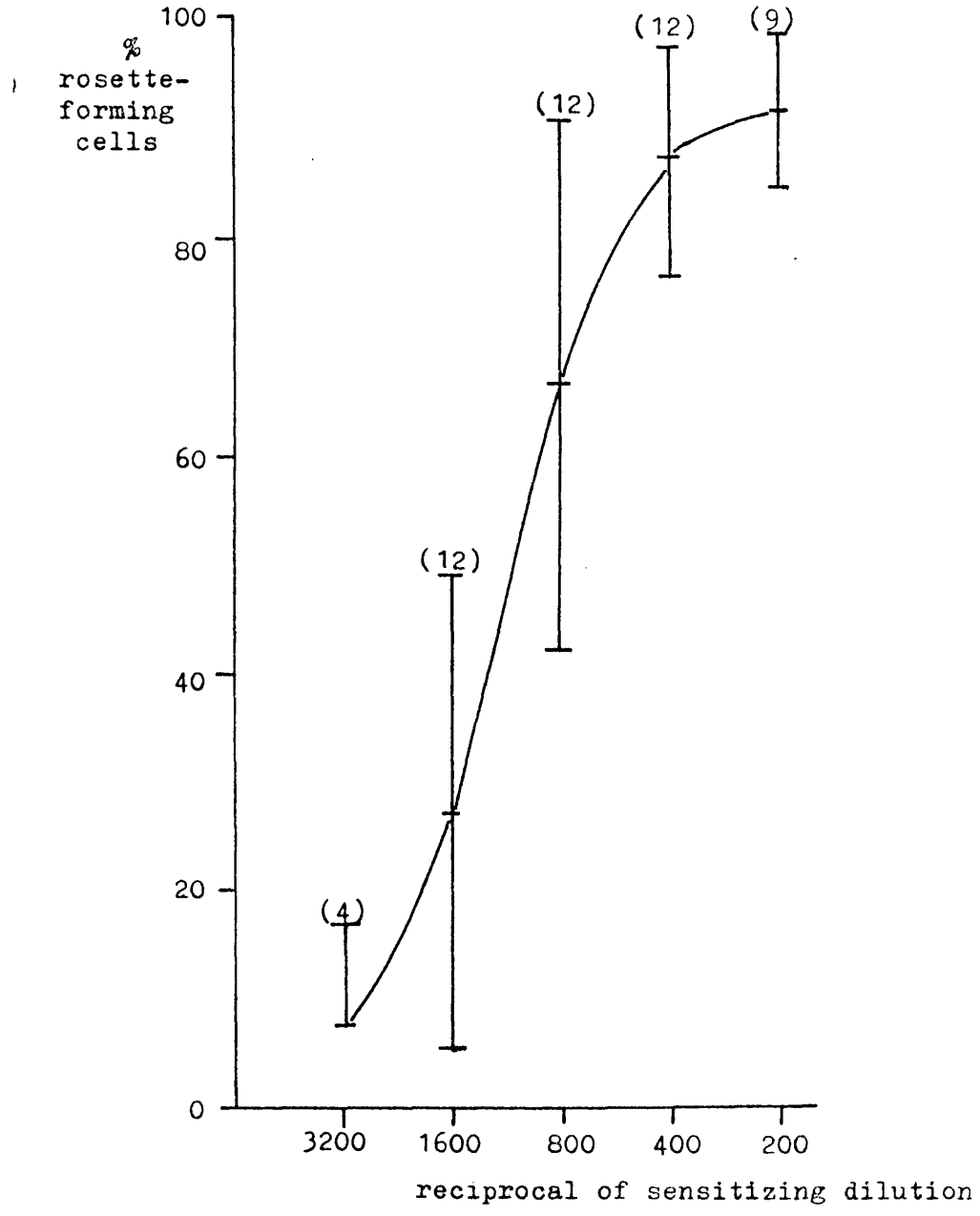


Figure 7.1

Rosetting behaviour of granulocytes from rheumatoid arthritis patients.

Results are expressed as $\bar{x} \pm 1$ SD with the number of patients given in brackets.

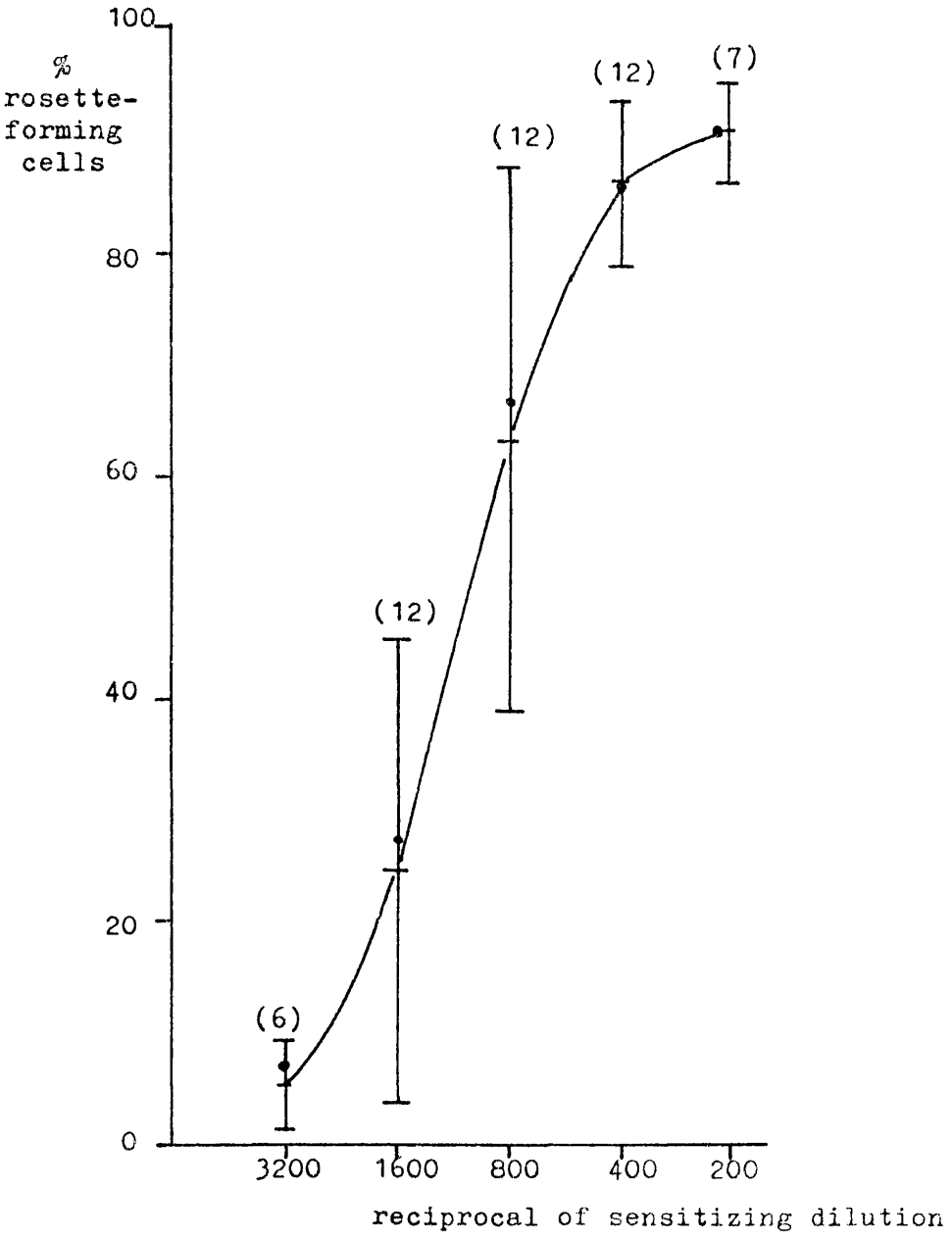


Figure 7.2

Rosetting behaviour of granulocytes from normal subjects. Results are expressed as $\bar{x} \pm 1$ SD with the number of subjects given in brackets.

- represents the means from Figure 1 for comparison.

the uptake of the red cells, Neither of these theories was borne out since no difference between the two subject groups was demonstrated. Rhodes has observed that Fc receptor expression increases with macrophage activation and is above normal in patients with neoplasms (Rhodes, 1975 and 1977). Non-malignant diseases (except tuberculosis) caused neither an increase nor a decrease in this expression; five rheumatoid arthritis patients, for instance, showed enhancement in three and depression in two (Rhodes, 1977). The similarity in behaviour between monocytes of patients and normals in the phagocytic assay described in Chapter 5 reflects this finding.

Hall and Winrow have made a study of the IgG receptors on lymphocytes from a group of patients equivalent, but not identical, to those used in the assay described in this chapter. EA rosette formation by mononuclear cells was increased in RA patients over normals, being 21% compared to 13% for 1/800 coating, $P < 0.005$. When the red cells were coated with a limited digest fragment of IgG, termed Facb, rosettes were again formed more often in RA patients than in controls, and this was true whether or not the monocytes were removed by carbonyl iron treatment (Hall, Winrow and Bacon, 1980). The significance of these findings is as yet unestablished, but the difference between lymphocytic and neutrophilic rosette formation possibly reflects the very different roles of the cells in the immune response.

Since there appeared to be no distinction between the Fc_γ receptors on granulocytes, it was considered expedient to

study other properties of these cells that might have a bearing on the phagocytic response.

CHAPTER EIGHT FURTHER STUDIES ON PHAGOCYTES, II. BIOCHEMICAL EVENTS

Introduction

Phagocytic leucocytes are metabolically stimulated when they bind micro-organisms with the resultant production of microbicidal molecules such as superoxide ($O_2^{\cdot -}$), hydroxyl radicals ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) together with a transient light emission (Klebanoff, 1975; Rosen and Klebanoff, 1979; Easmon *et al.*, 1980). The same activation occurs whether the cell is exposed to chemotactic peptides (Kitagawa, Takaku and Sajamoto, 1980b; McPhail, Henson and Johnson, 1981), immunoglobulin aggregates (Henson and Oades, 1975; Treadway *et al.*, 1979), surface-bound immunoglobulin (Kiyotaki *et al.*, 1978) or zymosan (Doll, Wilson and Salvaggio, 1980; Easmon *et al.*, 1980; van de Stadt, van de Voorde-Vissers and Feltkamp-Vroom, 1980).

The majority of studies have been carried out on PMN leucocytes, for instance by Henson and Oades (1975); Rosen and Klebanoff (1979); Roos *et al.*, (1980) and McPhail, Henson and Johnson, (1981). Evidence supporting the presence of an equivalent system in mononuclear phagocytes has been provided by, for example, Kitagawa, Takaku and Sakamoto, (1980a and 1980b); Wilson, Tsai and Remington, (1980); Nakagawara, Nathan and Cohn, (1981) and Williams and Cole, (1981). This latter cell type, however appears to produce less of these highly reactive products than does the the granulocyte (Reiss and Roos, 1978; Johnson RB, Lehmeyer and Guthrie, 1976).

The interaction of the many metabolites generated during this process has already been outlined in the Introduction to this thesis (Figure 1.2, Chapter 1). Assays for measuring most of them are available and thus provide a means of assessing cell activation and, indirectly, phagocytic activity. The overall increase in hexose monophosphate shunt activity (with an increased oxygen consumption) has been known and measured for many years (Malawista and Bodel, 1967; Henson and Oades, 1975; Treadway *et al.*, 1979; van de Stadt, van de Voorde-Vissers and Feltkamp-Vroom, 1980). Measurement of the metabolites is dependent upon leakage of the active molecules or related enzymes into the extracellular milieu (Henson, Johnson and Spiegelberg, 1972; Root *et al.*, 1975; Babior *et al.*, 1981) and the efficiency of detection is influenced by this.

Small quantities of H_2O_2 in solution can be detected by the horseradish peroxidase - mediated oxidation of scopoletin which fluoresces only in its reduced form (Root *et al.*, 1975). Singlet oxygen oxidises methionine (Tsan and Chen, 1980). Superoxide can function as a reductant, reducing ferricytochrome C (Rosen and Klebanoff, 1976; MacGregor, Macarak and Kefalides, 1978; Babior *et al.*, 1981), or nitroblue tetrazolium (NBT) (Wenger and Bole, 1973; Lambert, Roberts and Wright, 1978), or as an oxidant towards epinephrine (Rosen and Klebanoff, 1976). Lucigenin-dependent chemiluminescence appears also to be mediated by $O_2^{\cdot -}$, while the luminol-dependent emission seems to be a result of the activation of more than one molecular species (Easmon *et al.*, 1980; Williams and Cole, 1981b).

The oxidation of serum thiol groups by phagocytosing polymorphs has recently been delineated in this laboratory by Hall and Blake (1981); it too is multifactorial, being dependent upon H_2O_2, $^{\cdot}OH$ radicals and possibly singlet oxygen ($^1O_2$). Serum sulphydryl levels are depressed in patients with active RA (Lorber *et al.*, 1964; Haataja, 1975) and rise with good patient response to anti-rheumatic drug therapy (Hall, Blake and Bacon, 1981). Hall and Blake (1981) suggest that this change could be related to free radical scavenging associated with phagocytic activity towards immune complexes which are decreased after chemotherapy.

The independence of the metabolic stimulation from exocytosis (Goldstein *et al.*, 1975) facilitates investigation of the effects of small macromolecules such as rheumatoid factor and soluble aggregates. It also allows direct comparison of cellular activation and measurable degranulation: in the light of the finding that rheumatoid peripheral blood granulocytes are less able to degranulate than their normal counterparts (see Chapter 5), it was considered of value to correlate this result with that of free radical release.

There appears to have been very little work carried out on the oxygen species released by rheumatoid phagocytes. Van de Stadt and co-workers could find no difference in oxygen uptake as compared to control granulocytes (1980), and the reduction of NBT appears to be normal (Wenger and Bole, 1973; Lambert, Roberts and Wright, 1978). Free-radical mediated peroxidation of serum and synovial fluid lipids has been studied with reference to RA and other

arthritic conditions. Synovial fluids from inflammatory diseases (mostly ankylosing spondilitis and RA) had a higher level than non-inflammatory arthritides (most osteoarthritis);and the serum peroxidation of RA patients was raised above normals (Lunec *et al.*, 1981). This is an overall response, and the authors noted that "the specificity of the changes in RA therefore needs to be defined".

In consideration of the changes in serum thiol levels in RA, it was decided to use their oxidation as a measure of phagocytic activation. Since this assay appears to be independent of superoxide,it was thought expedient to assess this moiety separately using the ferricytochrome C assay. The major features of this section are, therefore, as follows:

One: the comparison of granulocytes from normals and RA patients for superoxide (ferricytochrome C), other radicals (Thiols) and β-glucuronidase release.

Two: the assessment of superoxide and β-glucuronidase release from both monocytes and granulocytes exposed to soluble aggregate, particulate aggregate (HAGG), rheumatoid factor and synovial fluid complexes, either singly or in combination.

Related aspects are presented and discussed.

Methods and Materials

1. Patient and control groups.

Thiol oxidation experiments: Ten patients were studied, 4 M

and 6 F, age range 40 - 77 (mean age 56 years). All had synovitis, and one also had nodules and fibrosing alveolitis. Eight were seropositive and two seronegative. The mean duration of disease was 13.6 years, range 9 months to 35 years. No patient of this or following groups was being treated with second-line, anti-rheumatic therapy.

Nine controls volunteered, 2 M and 7 F, age range 20 - 59 (mean age 40 years).

Ferricytochrome C experiments: First group:

There were ten patients, 1 M and 9 F, age range 45 - 72 (mean age 58 years). All had synovitis, one also had severe erosions (ACA of 1/4, latex 1/4096), and one fibrosing alveolitis. Nine were seropositive, one seronegative. The mean duration of disease was 5.4 years, range 5 months to 15 years. Twelve controls were used for comparison: there were 3 M and 9 F, age range 20 - 59 (mean 37 years).

Second group: (buffer experiments)

Nine patients, 3 M and 6 F, had an age range of 35 - 74 (mean age 55 years). Seven were seropositive, one seronegative and one unknown. The mean disease duration was 10.4 years, with a range of 9 months to 35 years.

The controls consisted of 1 M and 6 F (one used twice), age range 25 - 60 , mean age 43 years.

Third group: (Modulation experiments)

This group was made up entirely of normal controls and was composed of 6 M and 4 females.

2. Methods

Granulocyte and mononuclear cell suspensions were prepared as described previously (Chapter 3, see Figure 3.3). The proteinaceous reagents, that is, rheumatoid factor (MxRF), soluble aggregate, HAGG and synovial fluid complexes were as detailed in Sections 2.3 and 2.5 as were the conditions for phagocytosis of HAGG leading to release of β-glucuronidase. In some of the experiments the granulocytes were suspended in buffered Earles Balanced Salt Solution without phenol red (EBSS), and the reagents in M 199 plus 10% FCS, so that the concentration of FCS was effectively reduced to 4 - 5%. The implications of this are presented in the Results section.
All tests were carried out in duplicate.

Oxidation of thiols by activated granulocytes:

The incubation conditions for this assay were, cells at 2.5×10^6 ml^{-1} in EBSS, HAGG at 200 µg ml^{-1} and as a source of thiols (SH) normal human serum (50 µl) to a total volume of 760 µl. After an incubation time of 2 to 30 minutes in a 37°C water bath, 40 µl of catalase at 3 mg ml^{-1} (Thymol-free, from bovine liver, Sigma) was added to decrease the chance of further oxidation occurring (Hall and Blake, 1981). The reaction tubes were cooled in crushed ice before being centrifuged at 1000 g for 5 minutes at 4°C in a temperature-controlled centrifuge. The SH assays were carried out as described by Hall and colleagues (Hall, Blake and Bacon, 1981), adapted by them for cell supernatants (Hall and Blake, 1981). Seven hundred microlitres of the supernatant was added to 100 µl of 0.1 M phosphate buffer pH 7.4, followed

by 200 µl of DTNB (5,5' dithiobis (2-nitrobenzoic acid); Sigma) at 0.8 mg ml^{-1} in 0.1 M phosphate buffer pH 7.4. After five minutes at 37°C, the absorbance of the reaction mixture was determined at 440 nm in a Cecil spectrophotometer. This was converted to serum SH levels (as µmol l^{-1}) using a factor derived from a calibration curve (Hall, Blake and Bacon, 1981), taking into account the 7 in 8 dilution of the cell supernatant. SH levels from serum samples were determined likewise (50 µl serum, 750 µl buffer and 200 µl DTNB).

Superoxide generation:

Granulocytes were studied at a concentration of 5 x 10^6 ml^{-1} and mononuclear preparations with the monocytes at 2 or 2.5 x 10^6 ml^{-1} (average 27% monocytes). The cells were incubated with various stimuli in the presence of 150 µM ferricytochrome C (type III, from horse heart, Sigma) as suggested by Roos and colleagues (1977), in a final volume of 250 µl, the diluent being EBSS. Each test was set up in quadruplicate; two for a direct determination and two containing, in addition, superoxide dismutase (SOD) at 10 µg ml^{-1} in order to determine the level of non-specific cytochrome C reduction (Goldstein *et al.*, 1975). After incubation in a 37°C water bath, usually for 30 minutes (see Results), the reaction mixtures were spun at 1000 x g for 10 minutes at 4°C in a temperature-controlled centrifuge. One hundred microlitres of each supernatant was then removed into 1.1 mls of 0.1 M phosphate buffer pH 7.4 (Goldstein *et al.*, 1975) and kept for up to two hours at 4°C. The absorbance at 550 nm was then measured in a Cecil spectrophotometer against a blank incubated without cells. Separate tests, also without cells, were treated

with potassium ferricyanide (final concentration 1.5 x 10^{-3} M) and with ferrous sulphate (Michelson, 1977) at 1.5 x 10^{-3} M, to determine the amount of cytochrome C that was reduced and the total amount present (reduced/oxidized respectively). The readings were converted to concentrations using an extinction coefficient of 21.1 $mM^{-1}cm^{-1}$ at 550 nm (van Gelder and Slater, 1962; Goldstein *et al.*, 1975), and expressed as nanomoles of ferricytochrome C reduced per 10^{6} phagocytes. The reduction in the presence of SOD was subtracted from the value in its absence to give the reduction specific to superoxide.

Results

3. Phagocytic oxidation of sulphydryl groups:

Preliminary experiments were carried out to standardize the thiol oxidation by granulocytes. Resting cells had no overall effect on the level of serum sulphydryls, whereas actively phagocytosing cells oxidized the thiol groups (Table 8.1). Titration of the aggregated IgG suggested that the response was maximal at 200 µg ml^{-1} (data not shown), so this concentration was used throughout. Time course experiments were carried out in a group of ten patients and nine controls, the data from which are presented in Figure 8.1. There was a consistent trend towards increased oxidation of the thiol groups by patients' PMN. However there was a high variability, especially amongst the control subjects, which diminished the statistical significance of this apparently increased oxidative activity. Only at 6 minutes was there a definite difference ($0.05 > P > 0.02$), while at 10 and 30 minutes significance was not established ($0.1 > P > 0.05$).

Table 8.1. The effect of granulocytes on serum thiol groups.

	Concentration of SH in serum ($\mu mol\ \ell^{-1}$)		
Subject	EB	BC	EL
serum only	604	554	670
serum + cells	541	583	710
serum + cells + HAGG	344	345	480

Table 8.2. The release of β-glucuronidase by granulocytes.

	% release above background (HAGG at 200 $\mu g\ ml^{-1}$)	
	$\bar{x}$	1 SD
Patients, n = 8	13.3	3.94
Controls, n = 7	12.8	3.06

Separate tests were also set up for β-glucuronidase release in eight of the patients and seven of the controls, and the results are summarized in Table 8.2. Surprisingly, there was no difference between the two groups. The correlation coefficient for β-glucuronidase release versus thiol oxidation was only 0.16 and not significant. Lactic dehydrogenase release was assessed for both thiol-oxidation and β-glucuronidase tests in nine of the subjects. The release was higher in the former (7.8 ± 5.4%) than in the latter (1.5%), after adjustment had been made for the presence of the fresh human serum.

4. Superoxide release by phagocytes:

Granulocytes from control volunteers were exposed to between 50 and 500 μg ml^{-1} of HAGG in the presence of ferricytochrome C. Maximal reduction took place between 200 and 400 μg ml^{-1}, and 300 μg ml^{-1} was chosen for this study. A time course experiment was performed: as shown in Figure 8.2 (two normals), the major part of the release of superoxide occurred before 15 minutes, with a smaller change thereafter. A thirty minute incubation period was chosen for routine use.

A study was made of twelve controls and ten patients with active RA, and the results are shown in Table 8.3. There were no demonstrable differences between the two groups for either superoxide or β-glucuronidase release, nor was there a significant correlation between the two variables ($r = 0.18$). The LDH release was higher than expected, being 11.4 ± 4.8% ($n = 15$). It was not obviously related to either background or stimulated superoxide

Legend to Figure 8.1.

The oxidation of thiol groups in serum by phagocytosing granulocytes.

$$\% \text{ SH groups oxidized} = \frac{\text{SH oxidized by cells responding to HAGG}}{\text{SH oxidized by resting cells}}$$

HAGG used at 200 μg ml^{-1}

Results plotted as mean plus or minus one standard deviation

▲ patient group

O control group

Statistical data:

time (minutes)	P value
2, 4, 8	not significant
6	$0.05 > P > 0.02$
10,30	$0.1 > P > 0.05$

Legend to Figure 8.2.

The reduction of ferricytochrome C by phagocytosing granulocytes.

Abscissa: Time of incubation

Ordinate: n moles of ferricytochrome C reduced by 10^6 PMN (reduction in the absence of SOD minus that in its presence i.e. superoxide specific)

● subject S.B., with HAGG

o subject S.B., without HAGG

× subject N.H., with HAGG

⊠ subject N.H., without HAGG

facing 138

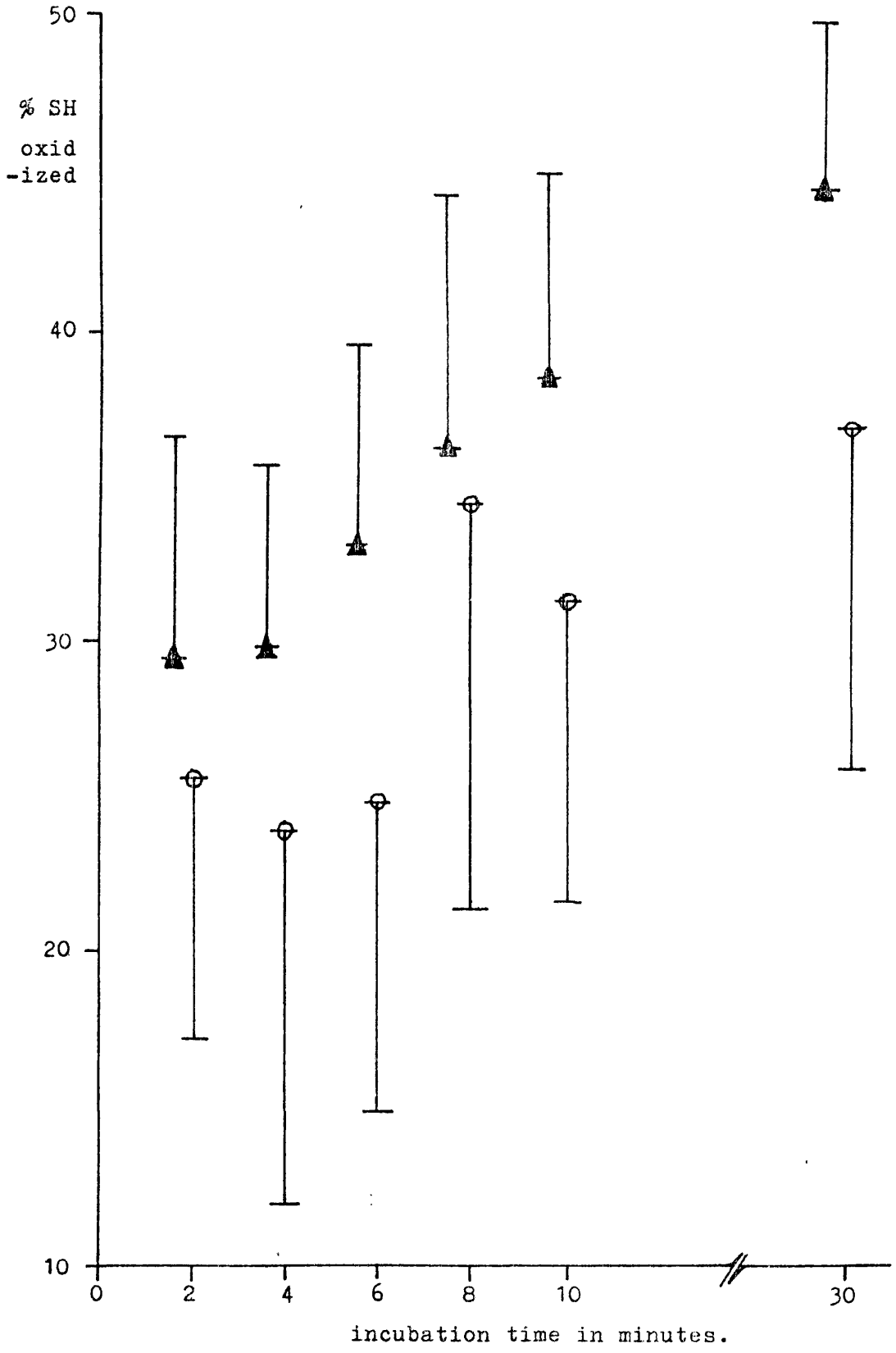


Figure 8.1

See legend opposite.

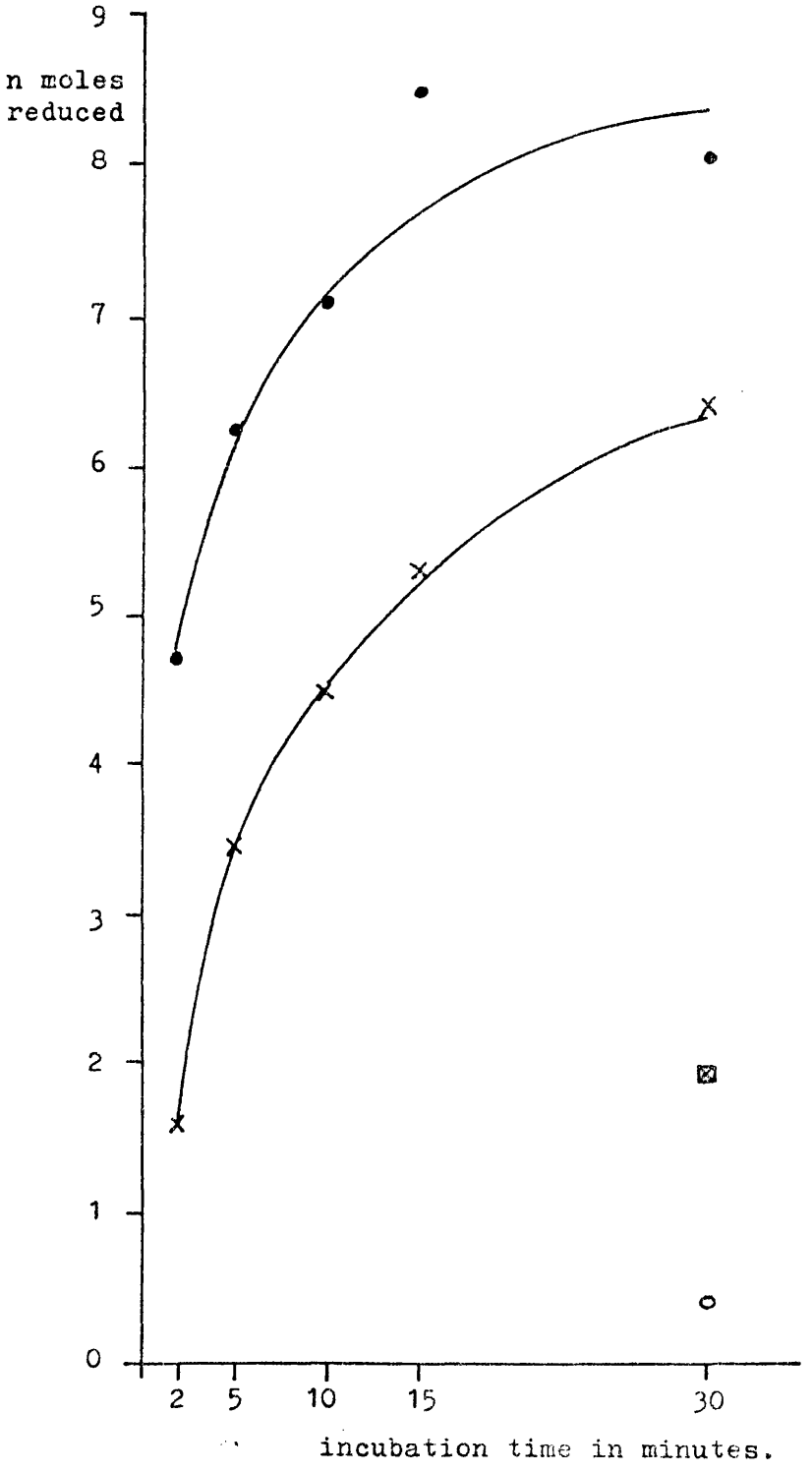


Figure 8.2

See legend opposite Figure 1.

Table 8.3. Superoxide and β-glucuronidase release by granulocytes.

n moles ferricytochrome C reduced per 10^6 PMN (superoxide specific):	Patients $\bar{x} \pm 1$ SD n = 10	Controls $\bar{x} \pm 1$ SD n = 12
without HAGG	1.09 ± 0.71	0.57 ± 0.87
with HAGG (300 μg ml^{-1})	4.33 ± 1.24	4.89 ± 2.29
change with HAGG (background subtracted at source)	3.24 ± 1.39	4.32 ± 2.52
% β-glucuronidase released (background subtracted at source)	21.56 ± 5.29 (n = 8)	21.80 ± 9.21 (n = 8)

release by the cells. The effect of an identical concentration of ferricytochrome C on the activity of purified LDH (type XII, from human erythrocytes) was assessed using the standard assay system, but no alteration of enzyme activity could be demonstrated.

5. Beta-glucuronidase release:

The discrepancy between the β-glucuronidase release demonstrated in these experiments and those described in an earlier chapter (Chapter 5, figure 5.1) necessitated a careful assessment of any changes that had been made in the procedure. The only difference was that instead of being suspended in M 199 plus 10% FCS, the granulocytes were in EBSS. The stimuli were, as before, diluted in the medium with serum, so that the final concentration was 40 - 50% M 199 plus 4 - 5% FCS. The effect of these changes was evaluated,using five patients and four controls, and Figure 8.3 depicts the results. The media had little effect except in the case of two patients whose cells responded much better in the EBSS/M 199 than in the M 199 alone.

It was noted that all controls were more active than patients, as had been found previously. In consequence four each of controls and patients had their neutrophils assessed for both β-glucuronidase and superoxide, and the results compared (Figure 8.4). There was again no correlation between the two indicators ($r = 0.26$), but patients showed a tendency towards a lower enzyme release. For statistical analysis, the data from this and the previous experiment (with media) were pooled; patients showed a lower release of the β-glucuronidase than did the controls, 21.4 ± 4.2% compared

Legend to Figure 8.3.

The effect of different media on the release of β-glucuronidase from granulocytes.

5 M - M 199 with 5% FCS

5 EM - M 199 with 10% FCS v/v EBSS (final FCS of 4-5%)

10 M - M 199 with 10% FCS

Stimulus 300 μg ml^{-1} HAGG

Legend to Figure 8.4.

Degranulation and superoxide release in phagocytosing granulocytes.

Ordinate: % release of β-glucuronidase (minus background)

Abscissa: n moles of ferricytochrome C reduced by 10^6 PMN

X RA patients

• Controls

Stimulus 300 μg ml^{-1} HAGG

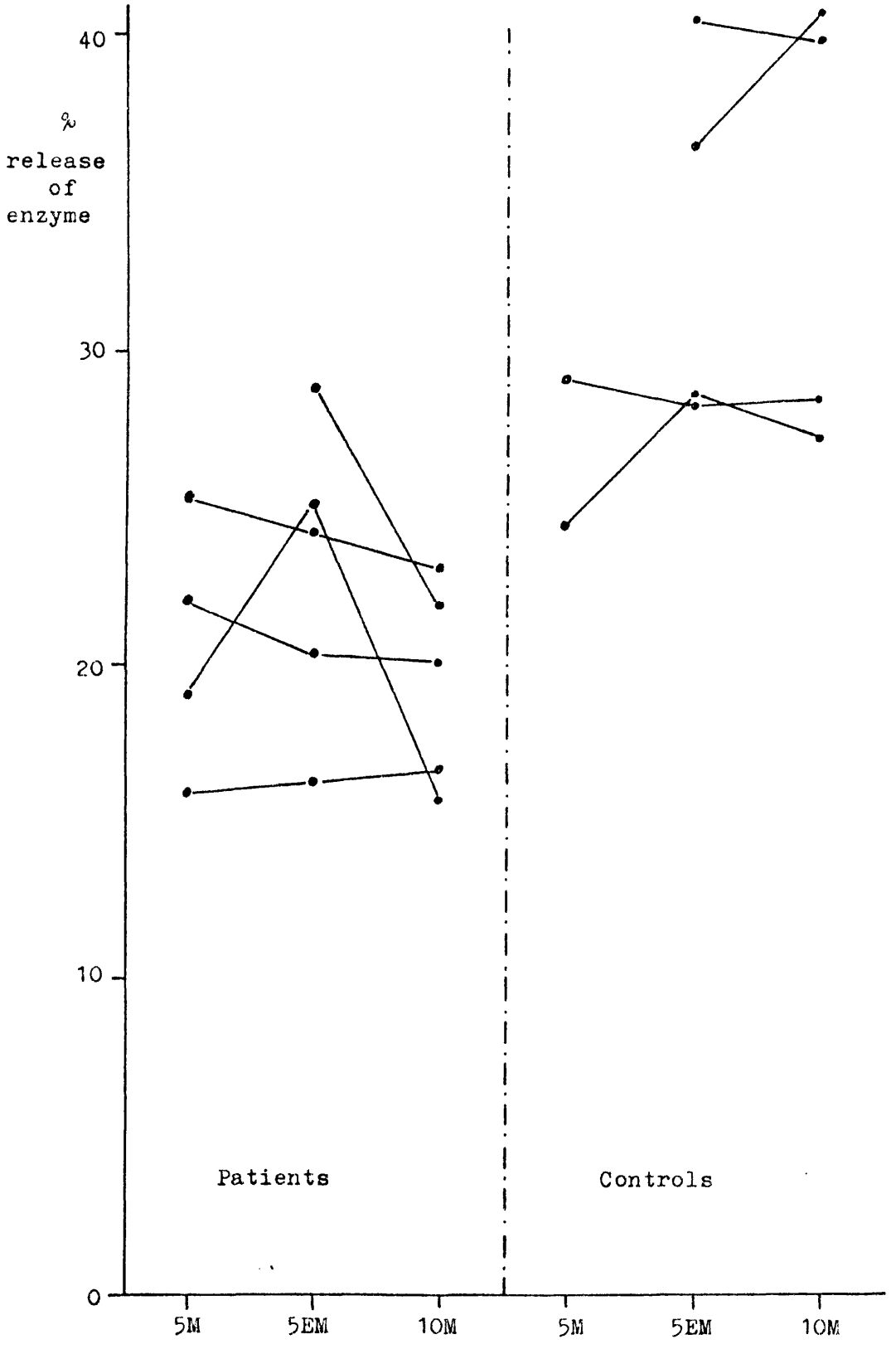


Figure 8.3

See legend opposite.

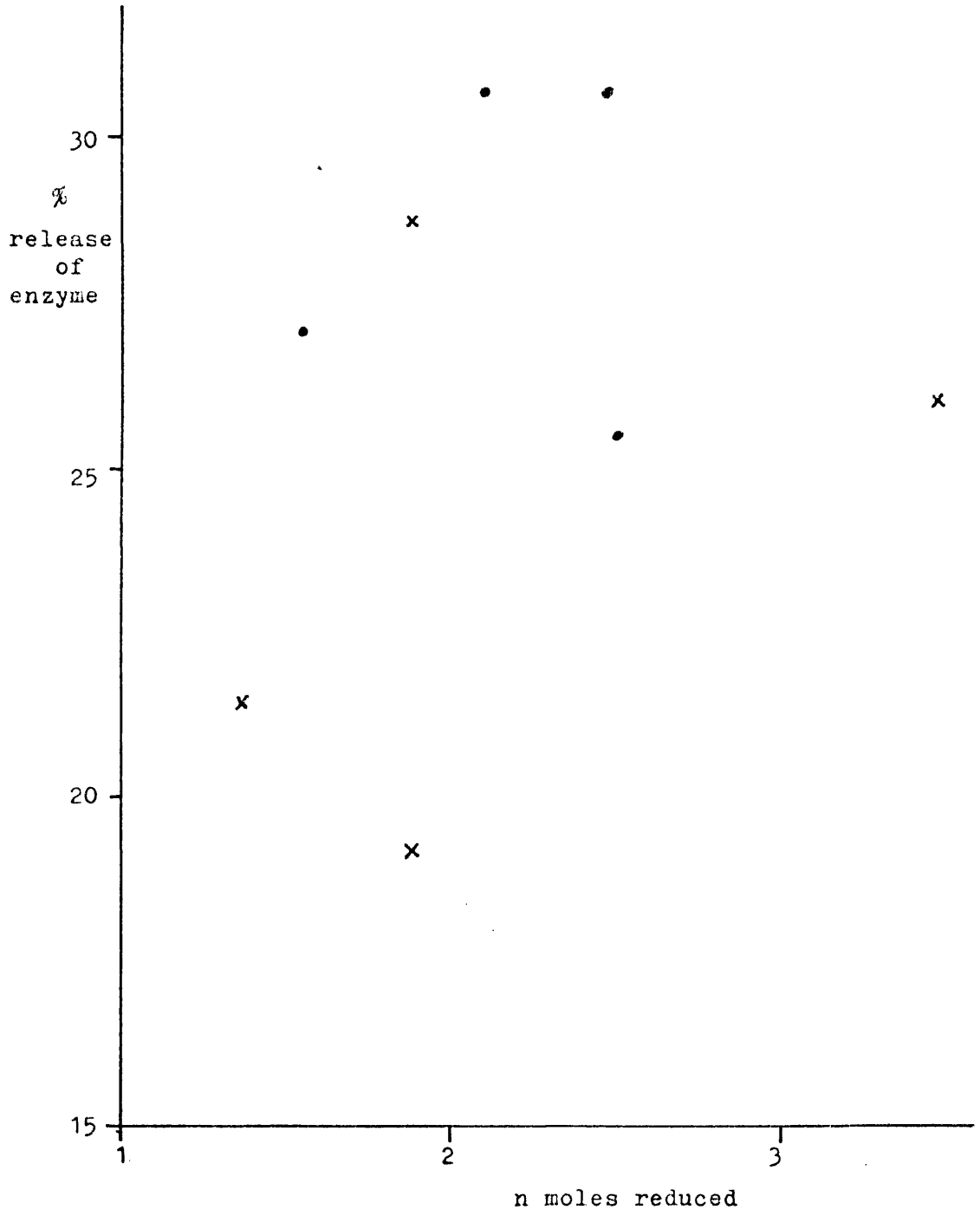


Figure 8.4

A comparison of enzyme and superoxide release by granulocytes.

See legend opposite Figure 3.

to 31.2 ± 5.8%, a ratio of 1 to 1.46 ($0.01 > P > 0.001$). This agrees closely with the data presented in Figure 5.1, where the ratio is 1 to 1.44. The difference in actual levels of release probably relates to the batch of aggregate used.

6. Inactivation of beta-glucuronidase:

Throughout the experiments in this thesis that involved measuring the acid hydrolases, it was noted that an overall decrease of enzyme activity occurred in stimulated phagocytes. That is;

$$\frac{\text{amount released + amount remaining (lysate)}}{\text{amount of enzyme in resting cell (lysate)}} = \text{Less than Unity.}$$

Because this may be related to effects of reactive oxygen species (Voetman *et al.*, 1981), the degree of change was calculated for the series of experiments summarized in Figures 5.1 and 5.2. The above equation was used with conversion to percentage activity by multiplying by one hundred. Similar results were obtained for each concentration of HAGG and representative data are portrayed in Figures 8.5 and 8.6. Both MNC and PMN showed the ability to inactivate β--glucuronidase though MNC were less able to do so than granulocytes. A difference between normals and RA patients was only demonstrated in one case, that of PMN responding to 50 μg ml^{-1} HAGG; rheumatoid phagocytes showed less inactivation of enzyme with a P value of < 0.05.

Five patients (not included in the above calculations) lacked the ability in one of the two cell types to induce this

Legend to Figures 8.5 and 8.6.

The inactivation of β-glucuronidase by phagocytes.

Figure 5 PMN
Figure 6 MNC
> RA Patients △
> Controls ○

Ordinate: % activity remaining:

$$\frac{\text{enzyme released + enzyme remaining in lysate}}{\text{enzyme in resting cell (lysate)}} \times 100$$

Statistical importance of the inactivation

P values:*	50	200	500 μg ml^{-1} HAGG
PMN patients		$P << 0.001$	
normals		$P < 0.001$	
MNC patients	$P < 0.001$	$0.01 > P > 0.001$	$0.05 > P > 0.02$
normals	$P < 0.001$	$P < 0.001$	$0.01 > P > 0.001$

*P calculated from $t = \frac{|\bar{X} - \bar{x}|\sqrt{n-1}}{s}$ $\bar{X} = 100$ $\bar{x}$ = % activity

symbol	patient	history
X	N.H.	Felty's syndrome. NSAID prior to Gold therapy.
▲	M.W.	Cyclic leucopenia. R_x of aspirin, amoxycillin and prednisolone 5 mg
●	C.B.	Definite RA with active synovitis. NSAID.
+	J.W.	Definite RA, psoriasis and active synovitis. Treated with NSAID.
⊠	E.C.	Definite RA with active synovitis. NSAID.

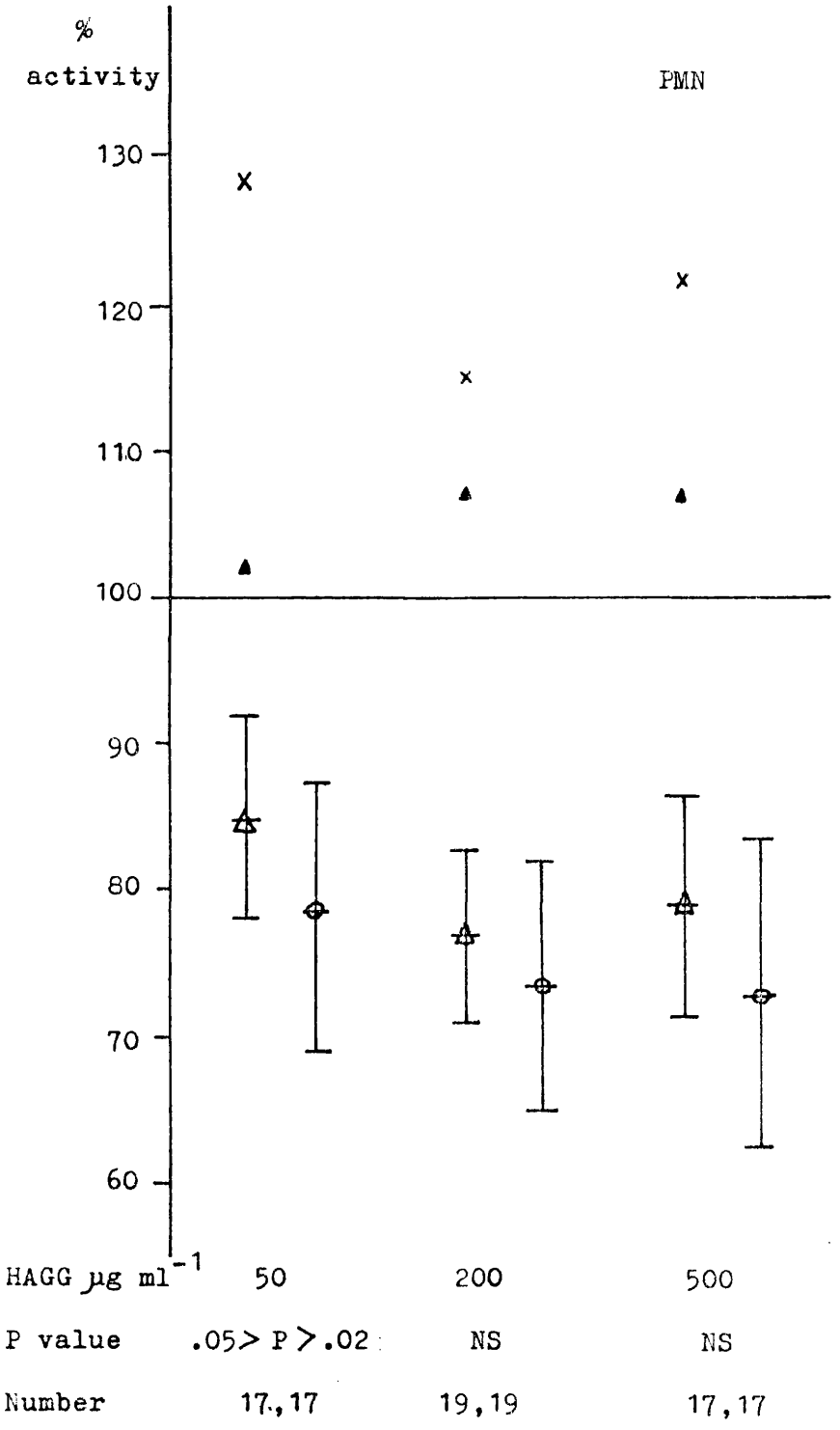


Figure 8.5 See legend opposite.

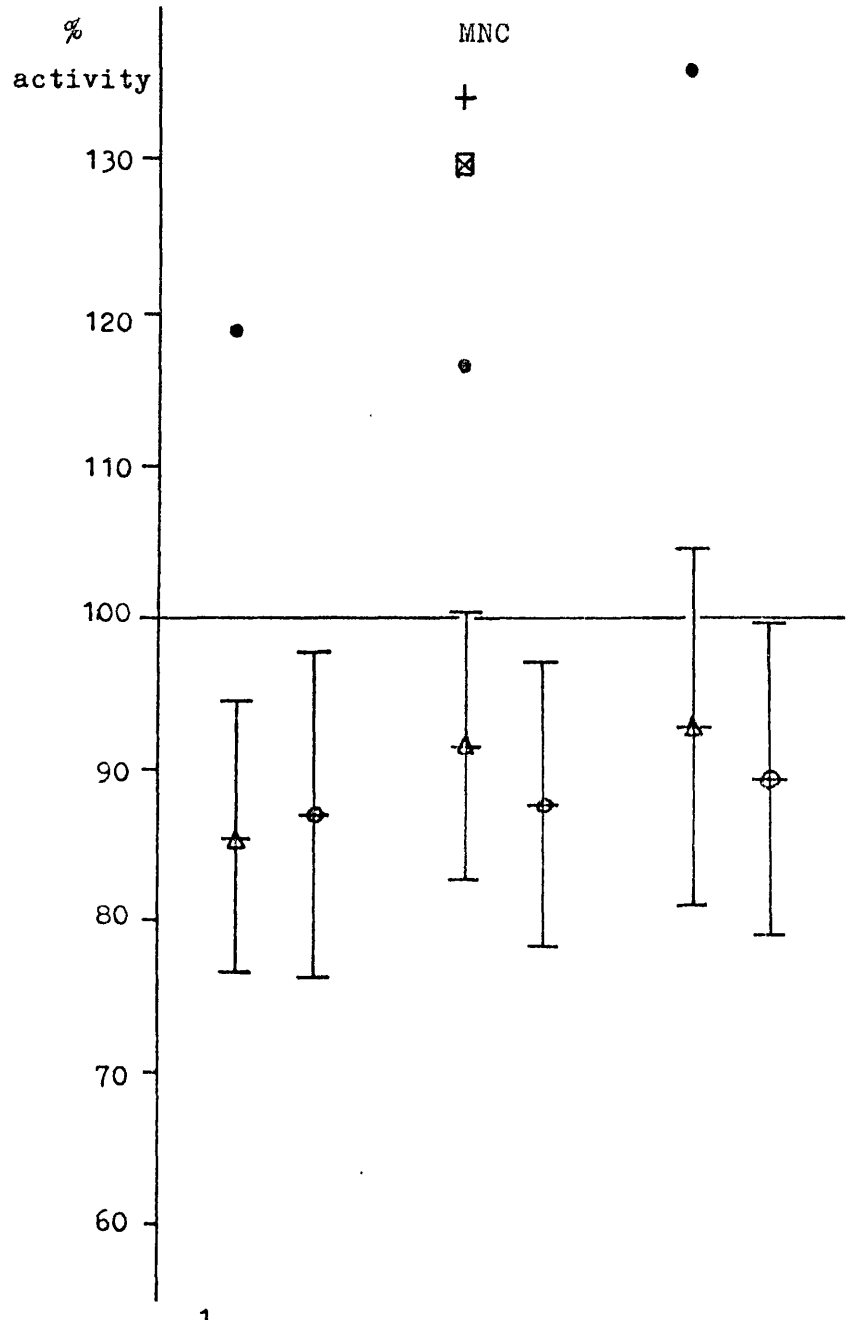


Figure 8.6

See legend opposite Figure 5.

change. None responded abnormally with both cell preparations. All showed adequate β-glucuronidase release.

Inactivation did not correlate with either thiols oxidized ($r = 0.09$, $n = 15$), cytochrome C reduced ($r = 0.25$, $n = 16$) or β-glucuronidase released ($r = -0.17$, $n = 19$ for patients, $r = -0.29$, $n = 18$ for controls) when granulocytes were involved. When the latter was calculated for MNC, the controls again showed a lack of dependency ($r = -0.28$, $n = 17$), while the patients cells appeared to demonstrate a positive relationship between inactivation and enzyme release ($r = -0.55$, $n = 17$; $0.05 > P > 0.02$). The coefficient r is negative because nil inactivation leaves 100% of the enzyme remaining, and total inactivation would stop all enzyme activity. The group of 4 patients and 4 controls also showed no correlation between the variables. Most of these calculations were carried out on data from experiments with 200 μg ml^{-1} of HAGG; reduction of cytochrome C with PMN was in response to 300 μg ml^{-1} HAGG.

7. Activation of phagocytes by small macro-molecules.

Ferricytochrome C reduction:

It has been determined that rheumatoid factor preparations, soluble aggregates and immune complexes from synovial fluid cause little, if any, degranulation by phagocytes (see Figure 4.4 and Chapter 6.6). Their ability to stimulate the cell, leading to superoxide release, was investigated. The rheumatoid factor preparation was IgG/IgM, designated as MxRF (Chapter 6); it reacted with mononuclear cells in 4 out of 6 cases, and with

granulocytes in 6/6 cases, although at a lower level. Soluble aggregates were as active in stimulating the cells as was the particulate HAGG, and the synovial fluid IgG (SF IgG) aroused a similar response. Subjects 1 and 3 demonstrated high release from resting neutrophils that was in excess of the level $\bar{x}$+1 SD (0.64 + 0.60) calculated from previous experiments, and this increase was reflected in almost all responses. Subsequent to the investigations on subject 3, the 'sterile' stock of EBSS was found to be contaminated and was discarded. It is probable that these abnormal results were a consequence of the presence of endotoxins, to which neutrophils were more sensitive than the monocytes. The background superoxide release was subtracted from that of the other test values for PMN, subjects 1 and 3. Although subject six' PMN generated superoxide in the resting state this did not raise stimulated values, so could not with validity be subtracted from them.

The results, as shown in Figures 8.7 and 8.8, indicated that it was difficult to be quantitative about the reduction of cytochrome C. The addition of two stimuli concurrently produced no consistent increase or decrease in the release of superoxide (Table 8.4) which reinforced this impression.

An attempt was made to ascertain whether or not a plasma containing immune complexes could stimulate the cells' oxidative metabolism, The plasma D.C. (described previously, Chapter 4; Materials and Methods) was compared to two normal sera that were ACA and RF latex negative with normal CH_{50}, and all were utilized at a final concentration of 50% in the assay, in place of a stimulus.

Legend to Figures 8.7 and 8.8.

The ferricytochrome C reducing ability of phagocytes in response to various stimuli.

Figure 7: PMN at 5 x 10^6 ml^{-1}

Figure 8: MNC, with monocytes at 2.5 x 10^6 ml^{-1} (controls 1-3)

2 x 10^6 ml^{-1} (controls 4-6)

Ordinate: n moles reduced per 10^6 cells (superoxide specific)

(30 minute incubation)

Absicissae: stimuli as follows:

None

HAGG at 200 µg ml^{-1}

500 "

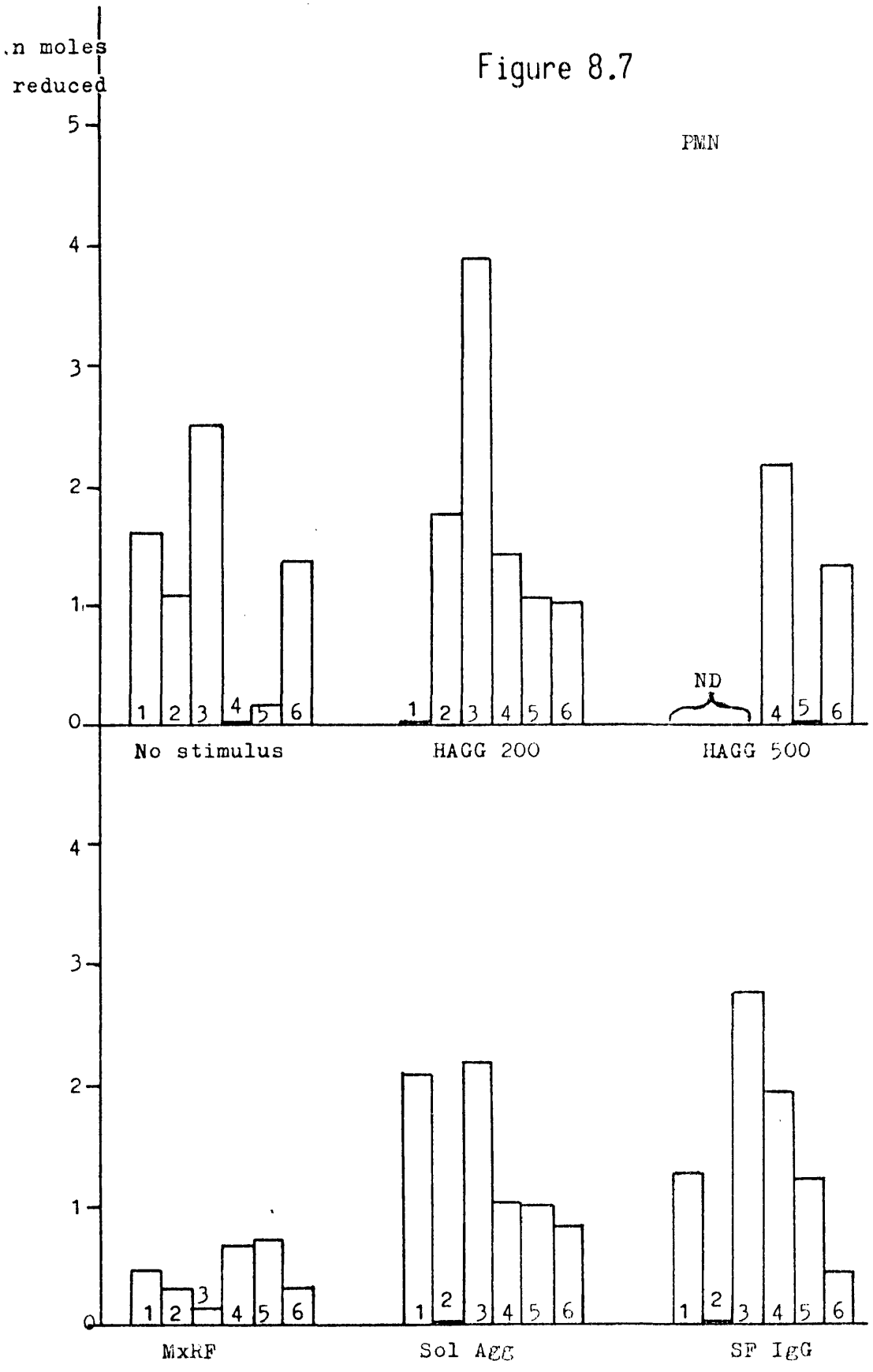
MxRF at 60 µg ml^{-1}

Sol Agg 500 µg ml^{-1}

SF IgG 500 µg ml^{-1}

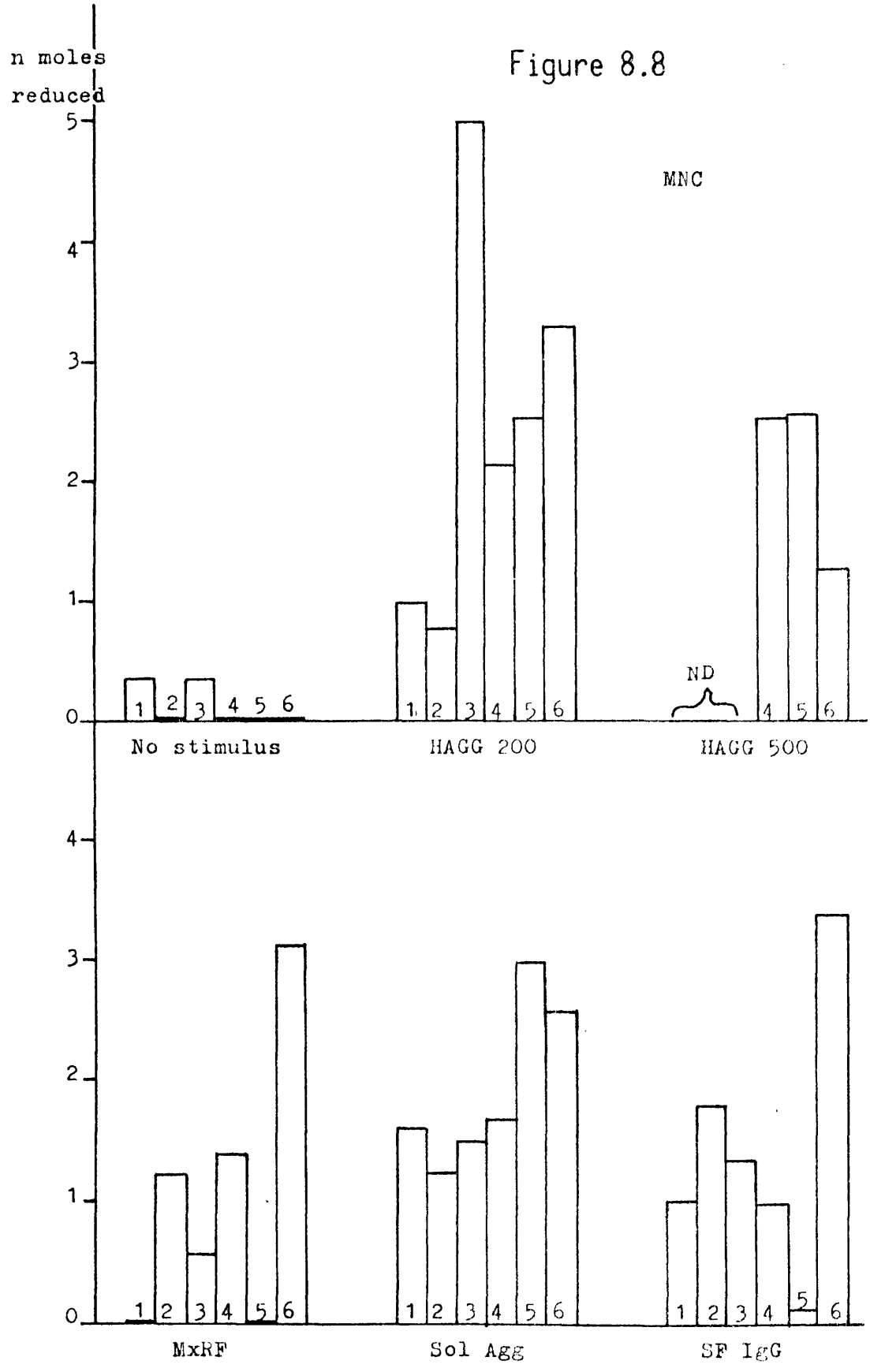
The numbers within the bars refer to the 6 control subjects used. For PMN, numbers 1 and 3 are presented with background (no stimulus) subtracted. See text.

Figure 8.7



See legend opposite.

Figure 8.8



See legend opposite Figure 7.

Table 8.4. The effect of concurrently administered stimuli on ferricytochrome C reduction.

Values equal $\frac{\text{n moles reduced in the presence of two stimuli}}{\text{n moles reduced in the presence of a single stimulus}}$

Numbers in parentheses refer to values with background subtracted.

Stimuli as in Figures 7 and 8 (HAGG at 200 μg ml^{-1})

SA = soluble aggregate

H = HAGG

	MNC				PMN	
Subject	$\frac{RF + H}{RF}$	$\frac{RF + H}{H}$	$\frac{SA + H}{SA}$	$\frac{SA + H}{H}$	$\frac{SA + H}{SA}$	$\frac{SA + H}{H}$
1	α	1.89	1.08	1.68	1.37 (1.61)	α
2	1.91	3.03	0.64	1.01	α	1.34
3	7.18	0.82	1.61	0.48	1.27 (1.58)	0.93 (0.88)
4	0.30	0.20	0.0	0.0	1.68	1.20
5	α	0.61	0.91	1.05	1.75	1.65
6	1.09	1.04	0.39	0.31	1.66	1.35
$\bar{x}$		1.27		0.76		
SD		1.03		0.61		

In 2 of the 3 granulocytes preparations, plasma D.C. induced more reduction of cytochrome C than did normal sera, while in the third the position was reversed. In addition all sera manifested an apparently SOD-resistant ability to reduce the cytochrome C, No firm conclusions could therefore be made.

Related experiments:

In conjunction with the investigation into the effect of rheumatoid factor and other compounds on production of superoxide, they were also judged for β-glucuronidase release with special reference to their modulation of the response to HAGG. It had been discovered that the rheumatoid factor containing both IgG and IgM (MxRF) had no effect on the response of PMN to HAGG, but decreased that of monocytes (Figure 6.1). It was decided to ascertain the properties of soluble IgG aggregates in this respect, and to compare them with the MxRF. Preliminary experiments with four controls in which soluble aggregate was titred against HAGG suggested that concentrations of 500 and 200 μg ml^{-1} respectively, gave readily detectable responses with both monocytes and neutrophils , (results not shown). MXRF was used at 60 μg ml^{-1} as in previous work. Cell concentrations were as in Figures 8.7 and 8.8.

The data presented in Figure 8.9 show that soluble aggregates do not influence the degranulation of neutrophils (effect, $\bar{x} = 1.1 \pm 0.2$). When mononuclear cells were tested, however, 4 out of 6 were inhibited by the presence of soluble aggregates, and 6/6 by the MxRF as expected (See Figure 8.10). There was a very

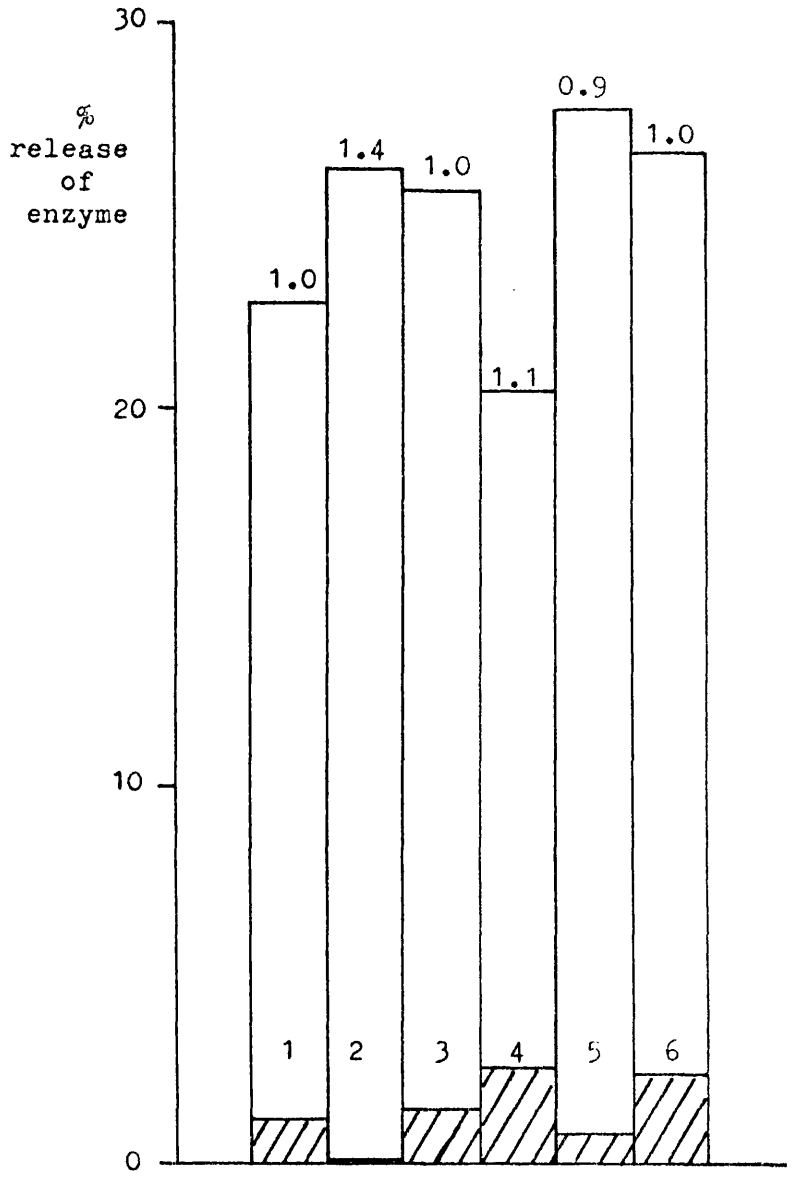


Figure 8.9

The interaction of granulocytes with soluble and insoluble aggregates of IgG: release of β-glucuronidase (background subtracted).

stimuli

open bars: 500 μg ml^{-1} Sol Agg plus 200 μg ml^{-1} HAGG

hatched bars: 500 μg ml^{-1} Sol Agg only

superscript numbers: ratio of release $\frac{\text{Sol Agg} + \text{HAGG}}{\text{HAGG alone}}$

The numbers within the bars refer to the 6 control subjects used.

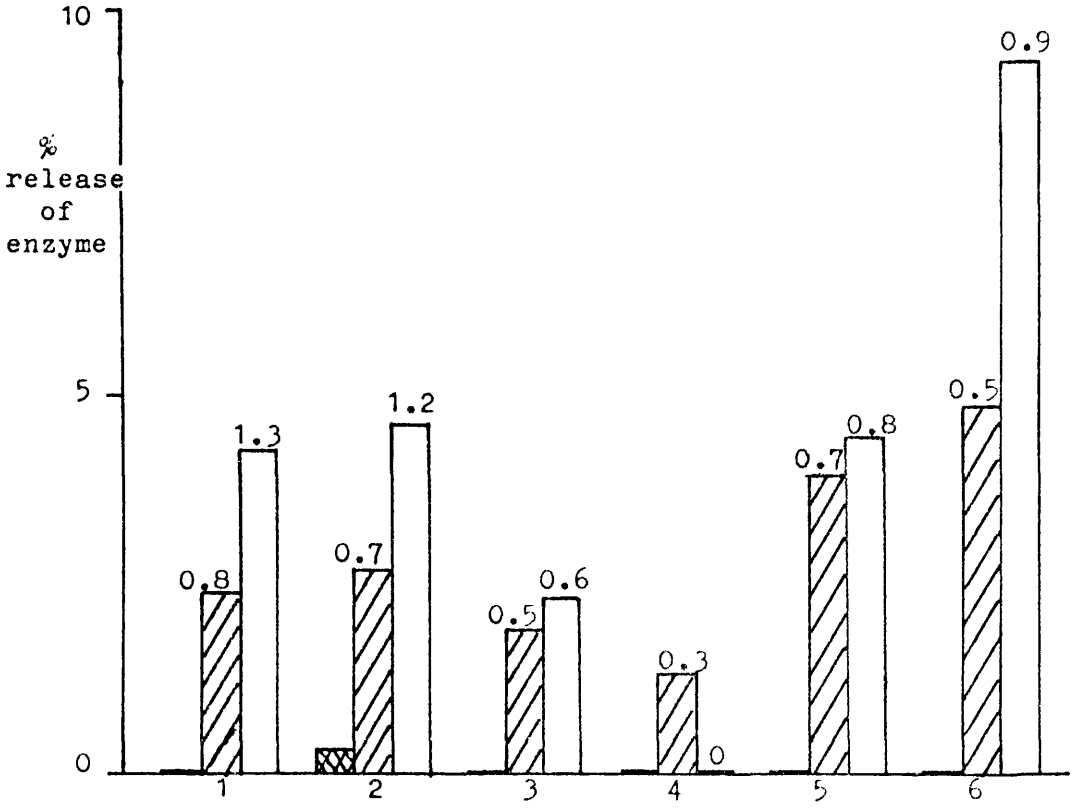


Figure 8.10

The release of β-glucuronidase by mononuclear cells (background subtracted) in response to rheumatoid factor (MxRF), soluble and insoluble aggregates of IgG.

	stimuli
open bars:	500 μg ml^{-1} Sol Agg plus 200 μg ml^{-1} HAGG
hatched bars:	60 μg ml^{-1} MxRF plus " " " "
cross-hatched:	500 μg ml^{-1} Sol Agg. No release except for subject 2.

superscript numbers:

ratio of release $\frac{\text{Sol Agg + HAGG}}{\text{HAGG alone}}$ open bars

$\frac{\text{MxRF + HAGG}}{\text{HAGG alone}}$ hatched bars

The numbers below the bars refer to the 6 control subjects used.

close correlation between the inhibitory efficiency of the two types of protein, and this is illustrated in Figure 8.11. The low P value strongly suggests that a common process was taking place.

The mixed rheumatoid factor did not induce inactivation of intracellular β-glucuronidase in monocytes (activity was 96 ± 15%, n = 4) or in PMN (100%, n = 2), and neither did soluble aggregates (103 ± 14%, n = 9; 109 ± 22%, n = 8, respectively) or SF IgG (100 ± 10%, n = 6; 106 ± 23%, n = 6). Similar calculations for the pure IgM RF used in previous experiments also indicated that this was the case (102 ± 8% for monocytes, 96 ± 10% for PMN), although the monocytes had not in this instance inactivated the enzyme in response to HAGG (97 ± 11%). The percentage activity left in the neutrophils exposed to HAGG was 69 ± 11%, which was close to that found in the preceding calculations.

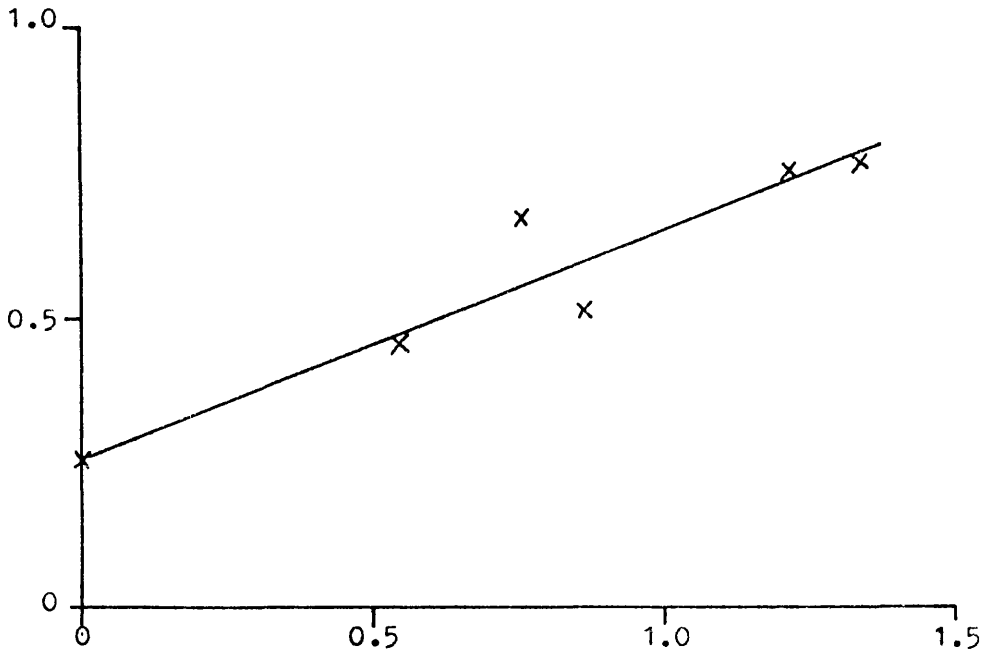


Figure 8.11

The relationship between the inhibition of response to HAGG by rheumatoid factor (MxRF) and soluble aggregates in mononuclear cells.

Y axis; $\frac{\text{MxRF + HAGG}}{\text{HAGG alone}}$

X axis; $\frac{\text{Sol Agg + HAGG}}{\text{HAGG alone}}$

The observed correlation is significant, $r = 0.94$ and $.01 > P > .001$

Discussion

The experiments presented in this chapter revealed three broad areas of interest. The two features of possibly lesser importance, that is the effect of serum on phagocytosis and the measurement of lactic dehydrogenase,are discussed prior to considering the release of oxidative molecular species.

8. The importance of the medium

The influence of serum on the phagocytosis of HAGG has been discussed, after the finding that the presence of heat-inactivated foetal calf serum increased the release of β-glucuronidase by PMN (Chapter 6; Table 6.2) from normals. The effective decrease of serum concentration to 5% made in these experiments, did not cause an equal decrease in release from controls and patients, so that the differential response found previously (Figure 5.1) was abolished. Neither was the effect unipolar within the groups (Figure 8.3). This unexplained reliance upon the presence of HI, or decomplemented, serum needs to be investigated. Had the tests which showed no difference between the two groups of patients and controls been performed in the presence of 10% FCS, a difference in response may well have been demonstrated. In fact, subsequent experiments did demonstrate responses which were in close agreement with the data presented in Figure 5.1 (see Results 8.3).

9. The measurement of lactic dehydrogenase.

The apparently high LDH activity found in the presence of ferricytochrome C is likely to be an experimental artifact, because its assay depends upon the disappearance of the added

cofactor NADH:

$$NADH + H^+ + \text{pyruvate} \xrightarrow{LDH} NAD^+ + \text{lactate}$$

absorbs at 340 nm

Ferricytochrome C can also be reduced by this compound:

$$NADH + 2 \text{ ferricytochrome C} \longrightarrow NAD^+ + 2 \text{ ferrocytochrome C} + H^+$$

(van Gelder and Slater, 1962)

The finding that an enhancement of NADH disappearance did not occur when only purified LDH was used strongly suggests that this second reaction was catalysed by something released by the cells into the supernatant.

Other experimenters have measured LDH activity in the absence of cytochrome C (Goldstein *et al.*, 1975; McPhail, Henson and Johnston, 1981), or have carried out a dye-exclusion test for viability after the assay itself (Kitagawa, Takaku and Sakamoto, 1980). This latter method seems to be the most promising in assessing the actual effect of ferricytochrome C on cell viability.

10. Oxygen-dependent products

Oxidative molecular species released from stimulated phagocytes were measured by their ability to oxidize serum thiols and to reduce ferricytochrome C. Serum sulphydryl levels are depressed in RA patients (Lorber *et al.*, 1964; Haataja, 1975), and since they can be oxidized by phagocytosing PMN (Hall and Blake, 1981), it was of interest to ascertain if rheumatoid granulocytes release relatively more oxidizing molecules than do those from controls. There were indications that rheumatoid

patients' cells did indeed oxidize serum thiol groups more readily than did controls' cells, but no firm conclusions could be drawn because the SH oxidation was found to be very variable (See Figure 8.1). Thus it seems likely that the decrease of reduced serum thiols to about 60% of normal levels (Lorber *et al.*, 1964) is owing, at least in part, to two factors. These are the apparent tendency for patients' cells to more readily oxidize thiols, and to the increased frequency with which the granulocytes are likely to be stimulated to release active oxygen species after binding complexes and the like deposited at vessel walls. In support of this, it has been reported by Lorber *et al.* (1964) that the most severely depressed serum sulphydryls are to be found in connective tissue diseases associated with vasculitis (e.g. rheumatoid arthritis complicated by vasculitis).

This assay system might prove useful towards deciding at which level the second-line drug D-penicillamine acts to effect a return to normal serum thiol levels (Hall, Blake and Bacon, 1981). Anti-inflammatory drugs have been shown to inhibit superoxide production (Oyanagui, 1978), and if they have similar action on the cells' ability to oxidize thiols this should be demonstrable using the described technique.

The kinetics of release of superoxide found in the present studies (Figure 8.2) (as measured by ferricytochrome C reduction) were very close to those demonstrated by both Simchowitz and Spilberg (1979) and Goldstein and colleagues (1975). The latter used aggregated IgG as one of the stimuli. Goldstein showed this

activity to be independent of β-glucuronidase release and this was also found to be true for the present experiments (Figure 8.4). This is possibly because the two are temporally distinct, the change in oxygen consumption occurs at particle uptake and closure of vacuole (about 20 seconds) while degranulation occurs at about 400 - 700 secs (Segal, Dorling and Coade, 1980). Degranulation need not follow the first event at all (Goldstein *et al.*, 1975; Reiss and Roos, 1978). In the light of this lack of correlation it was not unexpected to find that superoxide production was no different in rheumatoid and normal granulocytes (Table 8.3). The slightly increased background release of rheumatoid neutrophils was probably not important given the variability of all the results (see Table 8.3), and although it diminished the apparent release with HAGG, there was no significant difference between the groups.

The inactivation of acid hydrolases that occurred during phagocytosis was noticed early on in the present project. It is not a phenomenon commonly described, although at least one group of scientists have published their findings in relation to chronic granulomatous diseases (CGD) (Voetman *et al.*, 1981). Phagocytes from these patients did not inactivate their own enzymes, which strongly suggests that reactive oxygen products are the active agents. This is reinforced by the fact that inactivation did not occur under anaerobic conditions. The inactivation appeared to be caused by the combined action of myeloperoxidase and hydrogen peroxide (Voetman *et al.*, 1981). Almost all the rheumatoid patients' cells behaved comparably

with normals, only showing slightly less inactivation by PMN at one concentration, the significance of which was not established. Five of the patients behaved abnormally: two (both women with leucopenia) had granulocytes that failed to block their enzymes. One of the patients was definitely diagnosed as suffering from Felty's syndrome. Although their release of β-glucuronidase was apparently normal, and not increased as in CGD cells (Voetman *et al.*, 1981) it was at the upper limit of rheumatoid activity for both patients (17.3 and 18.7% c.f. 13.9 ± 4.0%, see Figure 5.1). It is therefore difficult to determine the effect this property is having on overall release. It would have been interesting to study this phenomenon in more patients suffering from Felty's syndrome, but many were already being treated with second-line therapy. One of the drugs within this group, notably D-penicillamine, has been shown to prevent superoxide-mediated changes in collagen and in hyaluronic acid (Greenwald, 1981) and to reduce the thiol groups in serum and glutathione in erythrocytes (Hall, Blake and Bacon, 1981; Munthe, Kass and Jellum, 1981). Munthe has shown this increase in reduced glutathione to also occur in response to some other anti-rheumatic compounds. It was therefore considered unwise to include such patients in the study. There were no specific features of rheumatoid disease in the three patients whose monocytes failed to inactivate their β-glucuronidase, for all had active synovitis as part of definite RA. Release of the enzyme was spread through the range of rheumatoids (16.6, 11.3 and 10.1 c.f. 12.9 ± 4.9%).

The apparent increase in activity of the acid hydrolase may point to the activation of latent enzyme within the stimulated cells. This would in turn mean that in the normal cell a greater inactivation is occurring than is directly measurable. This has not been reported in the CGD cells for β-glucuronidase, although a slight transient increase has been noted to occur with lysozyme (Voetman *et al.*, 1981).

It had been thought possible that the lower release of β-glucuronidase by rheumatoid granulocytes (Figure 5.1) might be a direct result of an increased inactivation, but this was not validated by the results obtained.

In the same way that active oxygen species are generated without a need for degranulation, phorbol myristate acetate, which induces almost no β-glucuronidase release, has been reported to promote inactivation of lysosomal enzymes (Voetman *et al.*, 1981). However, the soluble aggregates and mixed rheumatoid factor preparations used in the present studies failed to induce this alteration although both were capable of stimulating superoxide production. Elucidation of the exact mechanism whereby a cell induces inactivity in its own enzymes may help to explain these differences.

Monocytes have been reported to release less superoxide in response to zymosan than do neutrophils, primarily because seventy percent of the increased oxygen uptake in monocytes is utilized in mitochondrial respiration (Reiss and Roos, 1978). However the response to some formyl peptides, concanavalin A

and wheat germ agglutinin appears similar or higher in monocytes (Kitagawa, Takaku and Sakamoto, 1980). When paired granulocytes and mononuclear cells were used in this study (Figures 8.7 and 8.8) neither cell type showed a consistent increment over the other. However the inactivation of β-glucuronidase by monocytes (Figure 8.6) was less effective than that by granulocytes (Figure 8.5), which does suggest that the latter release more oxygen metabolites when stimulated by aggregated IgG.

When monocytes are cultured *in vitro* over four weeks they take on the morphological characteristics of macrophages, and by ten days retain less than six percent of their original capacity to secrete H_2O_2 and $O_2^{\cdot -}$ on challenge with opsonized zymosan or phorbol myristate acetate (Nakagaward, Nathan and Cohn, 1981). It is interesting that human alveolar macrophages are unable to produce luminol-dependent chemiluminescence, which has a similar dependency (Williams and Cole, 1981). The driving force for the monocytes to differentiate *in vivo* (and *in vitro*) is a property that the neutrophil lacks so that, teleologically speaking, the short-lived cell must act quickly with its bactericidal mechanism.

It was discovered in the work earlier in this thesis (Chapter 6) that granulocytes and monocytes behaved differently in the presence of rheumatoid factors. Because the inhibition of response to HAGG was only mediated by the MxRF, it was useful to ascertain whether this preparation contained self-associated complexes that were able to stimulate the phagocytes into their oxygen burst. This

was found to be the case. The MxRF promoted superoxide release in both monocytic and neutrophilic cells (Figures 8.7 and 8.8). The latter type appeared less easily stimulated. It was not possible to investigate the effect of the pure IgM RF because of lack of the reagent. Both phagocyte populations reacted to soluble aggregates of IgG and to the synovial fluid preparation, that contained putative complexes. The soluble aggregate behaved exactly as did the MxRF in that it produced an inhibition of degranulation to HAGG in monocytes, but not in granulocytes (Figures 8.9 and 8.10). The soluble aggregate was active only at a much higher concentration (500 $\mu g\ ml^{-1}$) than was the MxRF (60 $\mu g\ ml^{-1}$) and even so was less effective (Figure 8.11). This difference in effieciency parallels the finding that the inflammation properties of immune complexes depend very much upon their physical characteristics (e.g. Kavai *et al.*, 1981). Self-associated IgG/IgM complexes such as might be present in the MxRF would thus be expected to react differently, and probably more specifically, with the cells than would the soluble aggregates. Kavai and her colleagues (1981) and Griffin (1980) have both demonstrated that soluble complexes inhibit monocyte/macrophage phagocytosis, and more effectively at equivalence or antibody excess. This seems to be mediated by blocking or selective ingestion of Fc receptors (Griffin, 1980). The ratio of antibody to antigen within the MxRF would be complicated by the fact that the IgG molecules could act as both while the IgM is antibody only. In addition, the complexes could bind to either, or both, γ and μ receptors on the monocyte.

These experiments have shown that both MxRF and soluble aggregates are able to inhibit degranulation by monocytes and apparently by the same mechanism of reacting with the cells. It is likely that they block Fc receptors that would otherwise react with the stimulus (Arend and Massoni, 1981; Kavai *et al.*, 1981). Soluble immunoglobulin aggregates are taken up into lysosomes, so that exocytosis does not occur (Henson, 1977).

The same components were also able to cause superoxide release, but not inhibition of degranulation by PMN. Since these cells do not manifest IgM receptors (Henson, 1977), the response to MxRF must be mediated through the Fc γ portion. The formation of rosettes with lightly opsonized erythrocytes has been reported to be inhibited by aggregated IgG, not only for monocytes but also for neutrophils (Scott C.S., 1979; Kavai *et al.*, 1981). Binding to heavily sensitized molecules such as the particulate HAGG might be expected to be less easily inhibited. The Fcγ receptors on neutrophils have different characteristics from those on monocytes. For instance, they have a relatively low affinity for monomeric IgG as determined by inhibition of rosetting, and unlike monocytes can react, albeit weakly, with unaggregated IgG4 (Scott C, 1979; Lawrence, Weigle and Spiegelberg, 1975). They also require large amounts of particle-bound IgG as a stimulus for ingestion when complement is absent (Ehlenberger and Nussenweig, 1976 and 1977). Differences in affinities and concentrations of surface receptors are likely to be of the essence in a situation where response is so dependent upon a fine balance of the factors involved.

CHAPTER 9 PERSPECTIVES

A number of experiments have been performed in order to study the characteristics of phagocytes from normal controls and from patients suffering from rheumatoid arthritis. These tests are presented in Figure 9.1 in which the important results are also included. On the left of the figure is the effect of serum, the measurement of Fc receptors and the manifold study of granulocytes with respect to degranulation and membrane stimulation (Chapters 5 and 8; Results 8.3 to 8.6 and Discussion 8.10). On the right, the reactivity of monocytes and granulocytes in the presence of rheumatoid factors is presented.

It is interesting that the degranulation response of phagocytes is altered by the presence of serum whether it be human or bovine, fresh or inactivated for complement by heat or inulin treatment (Tables 6.1 and 6.2), and that this response appeared different in phagocytes from normals and from rheumatoid arthritis patients (Discussion 8.8). Which serum component is responsible for this behaviour is unknown and a search for it might provide some fascinating information on how granulocyte activity is regulated *in vivo*. It is also interesting to speculate whether the effect is upon β-glucuronidase itself rather than on the cells. The activity of this enzyme can be decreased at low protein concentration, and more specifically by organic acids related to the tricarboxylic acid cycle and by ascorbic acid (Pearse, 1972). Although it seems unlikely that such inhibitors are involved, weight should be given to them and their significance tested out.

Evaluation of the rosetting behaviour of control and RA's PMN was carried out (see Chapter 7) and no difference in their Fc_{γ} receptors could be demonstrated. The work laid out in Chapter 8 provides evidence that their ability to be stimulated into producing reactive oxygen species is similar in both groups. Rheumatoid phagocytes appear to secrete slightly more sulphydryl-oxidizing molecules but marginally less superoxide and hydrolase-inactivating activity (which is probably hydrogen peroxide/myeloperoxidase mediated, Voetman *et al.*, 1981).

The oxidation of serum thiol groups is thought to be mediated by hydroxyl radicals as well as by hydrogen peroxide (Hall and Blake, 1981) and the finding that this oxidation was slightly higher from patients' cells than from normals' neutrophils was in contrast to the significanct decrease in β-glucuronidase release. The events involved in the so-called metabolic burst are multifactorial (see for example Figure 1.2); it might be possible to discover whether one or more of the products could modulate membrane fluidity and phagosome-lysosome fusion. For instance, could the presence of larger than normal amounts of hydroxyl radical affect degranulation by neutrophils, and would the results of assays for this species correlate with β-glucuronidase release. $^{\cdot}OH$ promotes lipid peroxidation and so could damage lysosomal and plasma membranes (Lunec et al., 1981).

The transient life-span of such unstable molecules would seem to preclude their artificial addition to a phagocytic system. As an alternative, a generating system such as xanthine plus xanthine oxidase could be employed (Rosen and Klebanoff, 1979).

It is,however, equivocal whether the effects of such an external source could be compared to a reaction that apparently occurs close to the invaginating membrane of a potential phagosome. The study of cells from patients with congenital abnormalities of leucocyte function (e.g. by Roos *et al.*, 1980) has been invaluable in exploratory work and would be necessary in order to validate these proposals.

The normal Fc receptors and near-normal membrane stimulation (oxygen-dependent anti-microbial systems) but abnormal β-glucuronidase release raises the possibility that there is a lysosomal defect in the rheumatoid neturophils. Secondary lysosomes may not form as readily, so that a smaller proportion of enzyme is released. Alternatively, they may close off lysosomes more readily in the situation of frustrated phagocytosis than do normal PMN, so that the measurable degranulation is less.

Degranulation is only one part of phagocytosis, and if a particle is small enough to be taken into a phagosome, exocytosis does not occur. Such complete ingestion may have taken place to some degree with the particulate aggregate, but it was not measured. Other workers have done so using IgG-coated latex and yeast, but the results have been conflicting in that they show either a decrease or no change in PMN from rheumatoid arthritis patients in comparison to normals (Turner, Schumacher and Myers, 1973; Hallgren, Hakansson and Venge, 1978). It might be fruitful to measure, in separate assays, ingestion and degranulation of cells from the same blood sample. The relationship between the two

could be positively or negatively correlated. If, on the other hand, both non-phagocytosable and phagocytosable stimuli were put together, the cellular behaviour might reflect the results obtained for monocytes with the mixture of rheumatoid factors. It seems,therefore, that it would be reasonable to pursue these experiments further.

The total amount of β-glucuronidase within rheumatoid neutrophils is no different from that of normals (own observations, not shown; see also van de Stadt, van de Voorde-Vissers and Feltkamp-Vroom, 1980). It is conceivable that some β-glucuronidase may already be trapped with previously endocytosed immune complexes and be unavailable for release. The experimental finding that inactivation may not occur on uptake of small molecules indicates that the latter could happen without a decrease in the total enzyme content of the cell.

It would seem sensible to histochemically stain fixed cell preparations for the presence of β-glucuronidase. A method which detects solely lysosomal β-glucuronidase uses as a substrate Naphthol AS BI glucuronide and stains the enzyme red (Pearse, 1972). Subsequent treatment with a fluorescein or peroxidase-linked anti-immunoglobulin would allow parallel detection of immune complexes. Use of a horseradish peroxidase-linked "immunoperoxidase" method for detecting immunoglobulins could probably not be made because the brown deposits of its substrate could mask the red naphthol product mentioned above.

It is important to consider whether or not this abnormality of degranulation is confined to rheumatoid arthritis or whether it is found in other chronic diseases such as systemic lupus erythematosus or juvenile rheumatoid arthritis. If it is found to be widespread, the defect may be directly related to the uptake of immune complexes by the cells *in vivo*. The need to measure intracellular complexes as well as phagocytosis has already been acknowledged (Chapter 5, Discussion), and this would be carried out in any further studies. The techniques of immunofluorescence or immunoperoxidase-conjugated antibodies could be used to demonstrate the binding of antisera to such complexes in fixed cells. A parallel measurement of fluid-phase immune complexes might allow correlation of the two. However, since the results of the numerous tests for immune complexes vary considerably (see for instance, Soothill, 1977; McCarthy *et al.*, 1981; and Mumford, Horsfall and Maini, 1982), more than one would have to be carried out. The size of the complexes should be measured by, for instance, gel filtration or polyethyleneglycol precipitation, in addition to considering their biological properties of binding rheumatoid factor and Fc receptors.

It seems unlikely that granulocytes that have been circulating freely in the blood would contain immune complexes; only those that have adhered to sites of deposition would be expected to have ingested them. This opinion is borne out by the observations that peripheral blood PMN contain few or no inclusions as detected by immunofluorescence while larger numbers are found in those from synovial fluid to which the PMN have migrated in response to

inflammatory stimuli (Vaughan *et al.*, 1968; Cats, Lafeber and Klein, 1975).

It is not known how many of the cells adherent to vessel walls become free-flowing again once they have ingested immune complexes, and what proportion of these are taken up by the mononuclear phagocyte system on their first circuit through the liver. Pertinent questions such as these need to be answered before the effect of immune complexes on the neutrophil population as a whole can be understood.

The degranulatory response of monocytes to particulate aggregated IgG is inhibited if soluble aggregates of IgG, or both IgM and IgG RF, are also present (Figures 6.1 and 8.10). The equivalent activity in granulocytes was not, however, affected (Figures 6.1 and 8.9) although both cell types were activated to release superoxide. Possible reasons for this difference have been discussed in the previous chapter (Chapter 8).

The estimation of enzyme release in the presence of two competing stimuli (i.e. at the same time) is hardly likely to be measuring the same process as exposure of a cell to a stimulus *in vitro* some hours after it has possibly phagocytosed *in vivo*. An experiment in which phagocytes were pre-incubated in plasma containing immune complexes (from a patient with RA), followed only later by a challenge with aggregate, did indicate that ingestion of complexes could indeed decrease the degranulation activity of such cells (Figure 6.3). Unfortunately the tests

were inconclusive and much more work needs to be done in this field.

The relevance of both IgM and IgG rheumatoid factors to inflammation has been considered (Chapter 1); are they protective or pathogenic? At the concentrations used, pure IgM RF appears to neither stimulate nor decrease the activity of phagocytes towards the aggregated IgG used in these experiments. Higher concentrations of IgM RF would have been used had more been available; it may have blocked the activity of neutrophils towards the stimulus, as it has been shown to do in published work (Ward and Zvaifler, 1973b; Timms, Johnson and Henson, 1975), in effect by coating the stimulant with a protein for which the neutrophil has no receptor (IgM) (Henson, 1976). The effect of this preparation on monocyte degranulation is less predictable since IgM RF can bind to both aggregate and cell (see Discussion, Chapter 6).

The mixed IgM/IgG rheumatoid factor preparation, however, does block MNP degranulation and the close correlation between the inhibition by MxRF and soluble aggregates (Figure 8.11) suggests that the same process occurs. Like the soluble aggregates, the mixed-rheumatoid factor causes the release of free oxygen products that can alter molecules outside the cell (Lunec *et al.*, 1981). An additional consideration is that monocytes, destined to become residents of the mononuclear phagocyte system, aremade refractory towards an immunoglobulin-stimulus when both types of rheumatoid factor are present. This could mean that they clear circulating immune complexes less

well than if only IgM RF is present. *In vivo* therefore, this associated IgM/IgG could be pathogenic or protective. The finding of Allen and colleagues (1981) that these complexes bind complement and at high levels are associated with systemic manifestations of RA suggests that they are harmful rather than helpful.

A preparation that consisted solely of IgG RF was not available for investigation; IgM RF is ubiquitous which makes purification difficult. It would have been interesting to compare the effects of IgG rheumatoid factor with those of other preparations. If, for instance, IgG RF proved only as effective as the soluble aggregates at inhibition, then one could postulate that it is the presence of IgM RF within the complex that is of paramount importance. This would, in addition, explain the lack of effect on the IgM-receptor negative granulocytes. Such experimental use of IgG RF is clearly required if the mechanism of action of these anti-immunoglobulin antibody complexes is to be understood.

In relation to this, artificially prepared complexes such as BSA/anti-BSA could be usefully employed. Not only might they affect monocytes as the MxRF has been shown to do, but also their uptake by granulocytes might induce a decreased responsiveness such as that demonstrated for patients' cells.

The behaviour of rheumatoid arthritis patients' granulocytes was not apparently related to the administration of chemotherapy (Figures 5.3 to 5.5). This aspect could be investigated more

fully in patients who are not receiving any therapy, although this would be difficult. Alternatively normal volunteers could be 'treated' with a wide range of anti-inflammatory drugs for a longer period of time than that undertaken for the experiments represented in Figures 5.4 and 5.5. In addition to considering the effect of current treatment on laboratory tests, one should also take into account how therapy can be designed to bring those parameters back to normal in the hope that the clinical picture may improve. Biochemical improvements may precede those of the patient per se: for instance the levels of erythrocyte glutathione (reduced form) can rise by 20 to 40% prior to any evidence of disease remission induced by D-penicillamine or aurothiomalate (Munthe, Kass and Jellum, 1981). In a wider context, it would seem that if a drug is to be effective in combating chronic inflammation it needs to efficiently block one or more pro-inflammatory reactions such as those described in association with Figures 1.4 and 1.5.

The biochemical studies in this thesis have shown a significantly lower level of lysosomal enzyme secretion by rheumatoid neutrophils stimulated by particulate aggregated IgG, in comparison to their counterparts from normals. It is difficult to assess if the level of decrease found (for example 18.7 to 13.9%, a 26% decrease, at 200 $\mu g\ ml^{-1}$ HAGG), is clinically important. For instance, in a family which suffers from glutathione reductase deficiency, the heterozygous parents are healthy despite having lower than normal enzyme activity (Loos *et al.*, 1976b; Roos *et al.*, 1979). This situation is

also true of Factor VIII deficiency (haemophilia) in which patients with plasma levels exceeding just 5% of the normal are only mildly affected (Mollison, 1979).

It is possible that the decreased enzyme release might produce a tendency for internalized pathogens to survive, and to replicate within the host cell. This occurs as part of the normal life-cycle of some viruses within macrophages (Morahan and Morse, 1979). The process of lysogeny with occasional reversion to virulence is well-known in prokaryotic hosts (Lewin, 1974), and occurrence of this within the granulocyte could aggravate the situation. The effects of chronic viral infection with reference to immune complex disease have already been discussed in Chapter 1, not least the local tissue damage and autoantibody production so characteristic of the enigmatic disease of rheumatoid arthritis.

Rheumatoid arthritis is a chronic inflammatory disease in which a number of aberrations are evident,and it is to be hoped that as each researcher adds his or her piece, the circular jigsaw will be completed.

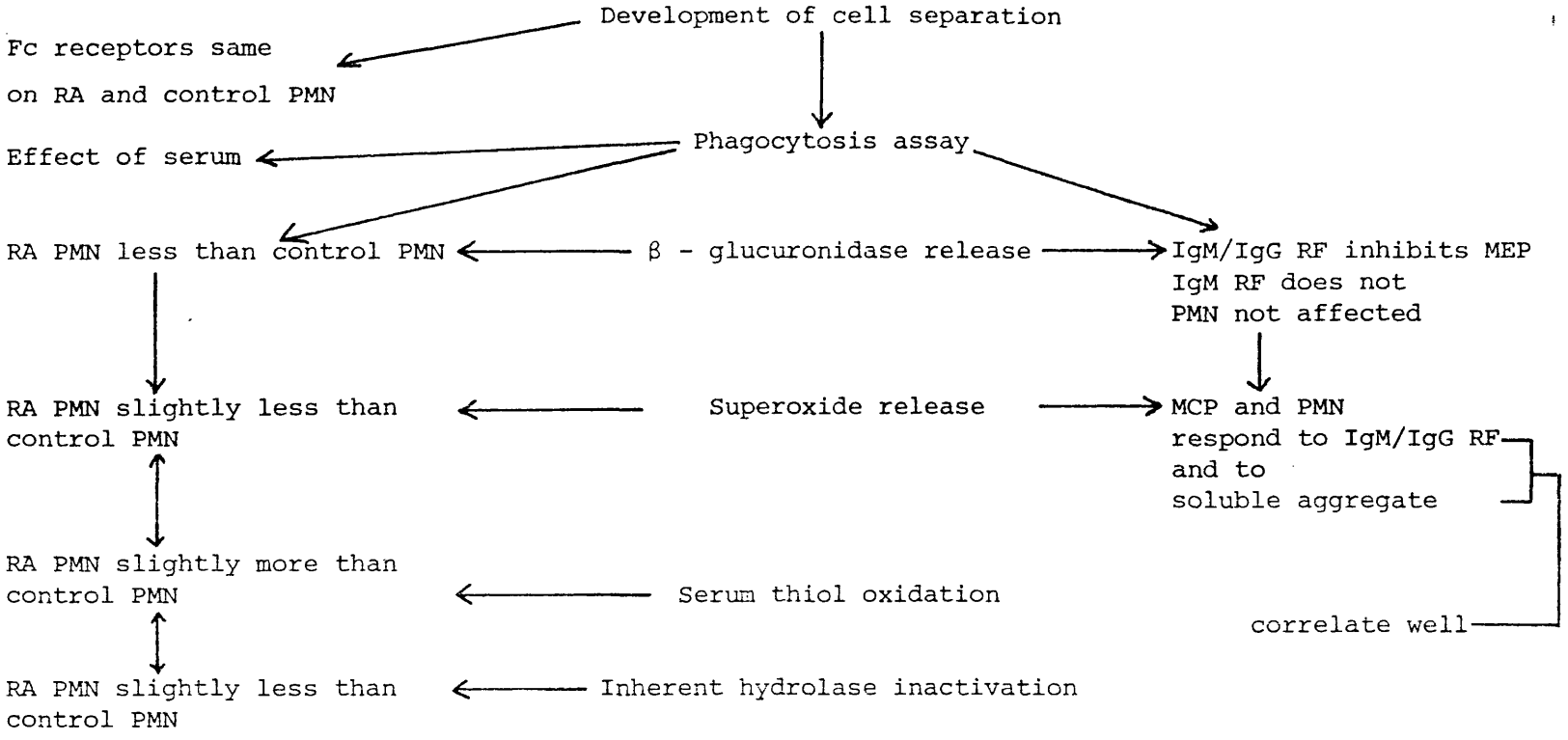


Figure 9.1 Summary of results (abbreviations as previously used)

I am gazing
Into what is man's foundation, the enduring scene that
stands,
Comforted by sun and water, glad of either in their
season,
Something that outlasts our minute, and has majesty for
reason,
While its granites wear to sands.

Much I wonder.
Much I long for this to speak, for an Incarnate Scene
to come
With a word for me to utter; so I ponder; so I hearken,
Watching closely that I see it ere my fading eye-balls
darken,
And repeat, ere I be dumb

All my knowledge,
All my being is summed up in this, the scene, that is a
friend
That is comfort through the evil and the agony of living
That, if heeding, and if judging, seems forgetting and
forgiving
And will seem so to the end.

Here I leave it.
Here it leaves me in the twilight, the imperfect wax it
prest;
Knowing this, that it has shaped me, or mis-shaped me,
for the telling
Of the purpose of the spirit that possesses this, in-
dwelling.
Knowing change, but never rest.

from 'The Hill' by John Masefield

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